PHD

Immune response to insulin in type 1 diabetic patients

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IMMUNE RESPONSE TO INSULIN

IN TYPE I DIABETIC PATIENTS

Submitted by
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SUMMARY

The cellular and humoral immune responses to insulin in type I diabetic patients (IDDM) were investigated in order to assess the immunogenicity of therapeutically administered insulin preparations.

In vitro lymphocyte proliferation (stimulation index (SI)) to human, pork and beef insulins was determined in 63 patients undergoing human insulin therapy. Approximately 40% of the patients gave significant SI values to at least one type of insulin. The cellular immune response to insulin was found to be partially regulated by suppressor cell activity.

The effects of transferring from beef to human insulin therapy was investigated in 31 patients. The type of therapy did not appear to influence the cellular immune response to insulin, implying that beef insulin was no more immunogenic than human insulin in these patients.

Serum immunoglobulin levels were determined in terms of anti-insulin IgG, anti-insulin IgG subclasses, total IgE and total IgM levels using an enzyme linked immunosorbance assay. Anti-insulin IgG was found to be significant in 55% of 76 patients undergoing human insulin therapy. Most responses were low; only 4 patients possessed high concentrations of antibody, all of whom had been administered PZI beef insulin at some time. Patients on human insulin therapy only showed no or very low levels of anti-insulin antibody in their sera.

Insulin therapy was found to stimulate preferentially IgG1 and IgG3 subclasses wereas the levels of IgG2 and IgG4 varied considerably from patient to patient. The levels of anti-insulin IgG4 and total IgE were found to correlate negatively with age and positively with the patients' daily insulin dose requirement. The level of anti-insulin IgG2 decreased with insulin dose. A strong correlation between anti-insulin IgG4 and IgE was also established.

Transferring patients from beef to human insulin did not have a significant effect on the levels of anti-insulin IgG or its subclasses. The levels of IgE, however, decreased when the patients were transferred to human insulin therapy.

CONTENTS

CHAPTER I

INTRODUCTION

Diabetes Mellitus is a disorder of metobolism associated with deficiency of insulin. The disease, which is characterised by hyperglycaemia, is chronic and affects the metabolism of carbohydrate, protein, fat, and electrolytes. The metabolic derangement is frequently associated with permanent and irreversible functional and structural changes at the cellular level. The vascular system is particularly susceptible.

Clinical complications of diabetes may arise. These characteristically affect the eye, the kidney and the nervous system. Examples of such complications include ketoacidosis, neuropathy, nephropathy, retinopathy, atherosclerosis and lowered resistance to infections.

Diabetes mellitus is the commonest of the endocrine disorders. In its various forms, diabetes afflicts about 5 per cent of the population in most western societies (Notkins, 1979).

On the basis of aetiology, two main categories of diabetes are recognised, namely primary and secondary diabetes. There are two main types of primary (idiopathic) diabetes: insulin dependent juvenile onset (IDDM) or type I diabetes; and a non-insulin dependent maturity onset (NIDDM) or type II diabetes mellitus. IDDM

usually occurs in childhood and adolescence, but it may also occur at all ages (Irvine, 1980). In Table 1.1. the main features of IDDM and NIDDM are compared.

Table 1.1.

Contrasting features of insulin dependent (IDDM) and non-insulin dependent (NIDDM) diabetes mellitus. (Modified from Reeves, 1980 and Rossini, et. al., 1985).

	IDDM	NIDDM
Age at onset -	Usually <30	Usually >40
Prevalence -	0.2-0.5%	2-4%
Onset -	Rapid or gradual	Insidious
Seasonal variation -	Present	? Absent
Insulitis at onset -	Present in 50-70%	? Absent
Insulin deficiency -	Absolute	Relative
Anti-pancreatic humoral immunity -	60-85% at onset	5%
Anti-pancreatic cell-mediated immunity -	35-50% at onset	<5%
Associated with other endocrinopathies -	Frequent	Infrequent
Concordance in identical twins -	<50%	Invariable
Association with HLA -	Present	Absent

IDDM is thought to be an autoimmune disease in which the beta cells of the pancreas are destroyed by inflammatory mononuclear cells which infiltrate the islets of Langerhans (Rossini, et. al., 1985). This process is referred to as "insulitis" and results in insulin deficiency and clinical diabetes. The immunological attack on the beta cells usually begins several years before the presentation of clinical IDDM and is referred to as the asymptomatic "prediabetic" period (Palmer,

1987). IDDM is thought to be initiated by virus, stress, environmental toxin or an aberrant immune response (Gepts, w., 1983). Antibodies directed against islet antigens are often found in the blood of newly diagnosed IDDM patients and in individuals during the "prediabetic" period. These antibodies include islet cell antibodies which are directed to the cytoplasm and cell-surface, antibodies to an islet protein of 64-KD molecular weight, and insulin autoantibodies (IAA) (Palmer, 1987).

Insulin autoantibodies are found in individuals who have not received exogenous insulin. Their characterization, shows them to be of the IgG isotype, with a similar binding constant and capacity to those of insulin antibodies from insulin-treated diabetic patients (Goldman, et. al., 1979) However, unlike the insulin antibodies induced by insulin therapy that have both lambda and kappa light chains, IAA from some patients with insulin autoimmune syndrome have been found to be exclusively kappa light chains (Palmer, 1987).

The role of IAA in IDDM, and whether it is pathophysiologically involved in the beta cell destructive process is unknown. It is possible that IAA may not be involved, but are only markers of the beta cell destructive process. IAA formation may be activated during the beta cell destruction process; IAA would then be considered a result of, rather than a cause of the beta cell damage. Alternatively, IAA may not be

necessarily markers of the beta cell lesion of IDDM, but may tend to occur in individuals genetically predisposed to autoimmunity, and a small proportion of these people may in turn be predisposed to IDDM. The finding of IAA in individuals with other autoimmune disorders such as Graves' disease, systemic lupus erythematosis and in individuals treated with drugs such as penicillamine suggest that IAA can occur and not be a marker of beta cell damage (Palmer, 1987).

The distribution of circulating lymphocytes may also be altered in IDDM patients. Although some investigations report normal numbers of peripheral blood B- and T-cells, others claim a decrease in the number of T-lymphocytes. Furthermore, the numbers of helper/inducer T-cells (OKT4, Leu 3A) have consistently been found to be unchanged when compared with controls. However, the numbers of peripheral blood suppressor/cytotoxic T-cells are reported by some workers to be decreased when compared with those of non-diabetic subjects (Rossini, et. al., 1985). Suppressor-T cell activity is also reported to be defective when lymphocytes from newly diagnosed diabetic patients are stimulated either by the mitogen concanavalin A (con. A) or by pancreatic antigens (Baschard, et. al., 1980). However, the suppressor cell activity is normal in established diabetics. This deficiency in suppressor cell activity is thought by some workers to contribute to the progression of the autoimmmune process in IDDM. IDDM shows a significant

assocuation with HLA antigens (see below), particularly with HLA-DR3 and HLA-DR4. An abnormal suppressor T-cell function has also been reported in non-diabetic subjects who are HLA-DR3 and/or DR4 positive. Therefore, an immune regulation abnormality of this kind may be related more generally to HLA-DR3 and HLA-DR4 (Rossini, et. al., 1985).

Interferon production (Baratono, et. al., 1980) and interleukin-2 (IL-2) (Kaye, et al., 1986) synthesis is also reported to be depressed in IDDM patients. Such defects do not appear to be related to the adequacy of metabolic control. However, it is possible that a cytokine required to modulate or suppress immunocyte activity may be deficient in IDDM. So far no evidence for such a hypothesis has been presented. Further investigation is therefore, necessary.

Pathogenesis of IDDM is thought to be genetically linked. The class II antigens HLA-DR3 and HLA-DR4 are present in increased frequencies in patients with IDDM in comparison with control populations (Wolf et.al., 1983; Irvine, 1980). Where both DR3 and DR4 occur together in the same individual, the risk of developing IDDM increases even further.

HLA-B8 and B15 antigens are also significantly associated with IDDM in caucasians (Irvine, 1980). A more variable association is observed with HLA-B18 and HLA-B40

(Cudworth and Festenstein, 1978), and HLA-B8 / W15 (Irvine, 1980). Diabetes associated with HLA-B8 and W15 is further characterized by early age at onset, non-obesity, lymphocyte infiltration of the islet of langerhans, reduction in the functioning beta cells and anti-pancreatic cell mediated immunity. The frequency of HLA-B8 is also reported to be high in Graves' disease and in idiopathic Addison's disease. This suggests the possibility of HLA-B8 or an HLA-B8 associated immune response gene being the common denominator for the development of endocrine autoimmunity (Irvine, 1980).

The possession of DR2, and/or HLA-B7 (which is often associated with HLA-A3) on the other hand, is thought to confer resistance to the development of IDDM.

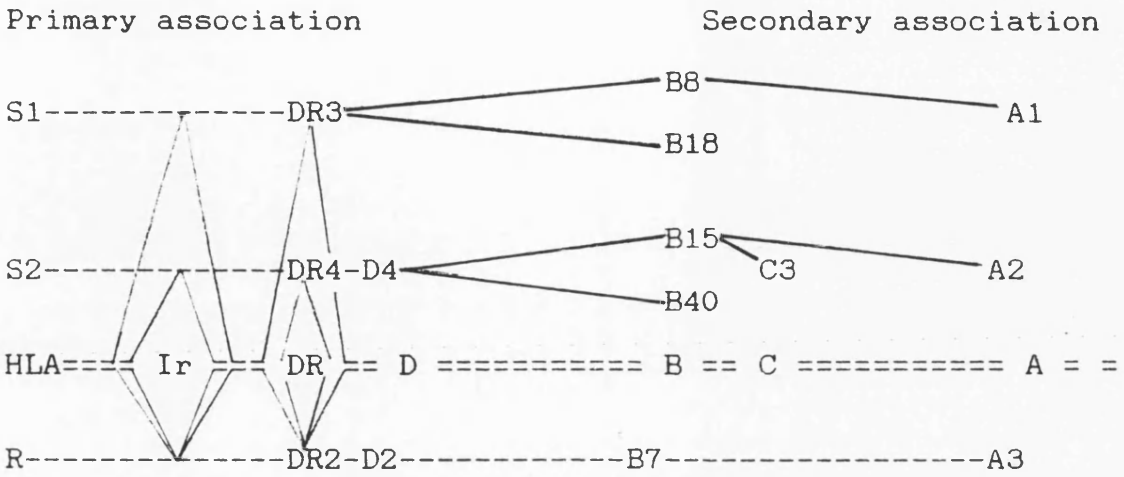
The HLA-D region has recently been studied in great detail using recombinant DNA technology. DNA probes and genomic blotting techniques have revealed five to six alpha genes, or heavy chains, (one DRa, three or four DCa related and one SBa). There are also seven beta genes (three DRb, two DCb and two SDb) (Steinmetz, M., and Hood,C., 1983). HLA-DC, which is in linkage disequilibrium with HLA-DR, is associated with IDDM, while HLA-SB is not (Rossini et. al., 1985). In fact, HLA-DC b-chain sequences are reported to be more strongly associated with IDDM than are HLA-DR antigens (Owerbach et. al., 1983).

The HLA genotype also appears to influence the patients'

age-at-onset before and after the age of 16 (Irvine, 1980). ie. there is a higher frequency of the genotypes DR4/x and DR4/4 and a lower frequency of other genotypes in patients with age at onset before the age of 16. Therefore, the presence of DR4 without DR3 appears mainly to increase the risk of developing IDDM before the age of 16, whereas DR3 increases the risk in all the age groups. This implies that DR3 and DR4 (or associated factors) act by two different mechanisms and that there are two peaks for the age at onset for IDDM, one at about the age of 12 to 14 years and one in the twenties.

Based on the above findings and the pattern of linkage disequilibrium within HLA, Cudworth and Festenstein (1978) have proposed a concept which involves double axes of susceptibility and a single axis of HLA factors conferring protection against the development of IDDM. This is illustrated in Figure 1.1.

Figure 1.1.



HLA axis of "Susceptibility" (S1 + S2) and "resistance" (R) to IDDM. (modified from - Irvine, 1980).

There is a progressive increase in relative risk from right to left along the HLA chromosome. The main susceptibility axis (S1) is defined by the primary association of DR3-D3, and the secondary association with B8, B18 and A1. The S2 axis is constructed in the same manner, DR4 representing the primary and B18, B40, C3 and A2 forming the secondary associations. The D(DR) determinants are probably in a stronger linkage disequilibrium with the gene which increases the predisposition to the disease. In contrast, subjects who are DR2, with a secondary association with B7 and A3, have a significantly reduced risk of developing diabetes. This is probably because of linkage disequilibrium with genes which confer protection, forming the resistance (or R) axis.

Recently, there has been growing evidence to suggest that a highly variable locus close to the human insulin gene on chromosome 11 may be associated with IDDM (Hitman, et. al., 1985; Neumer, et. al., 1988; Yokoyama, et. al., 1985). The IgG heavy-chain (Gm) gene complex has also been reported to be assocaited with IDDM and other autoimmune diseases (Nakao. et. al., 1981).

Thus the study of HLA asociations is beginning to shed light on the nature of an indiuvidual's proneness to develop insulin dependent diabetes. Genes associated with particular HLA phenotypes may determine the degree of immune responsiveness to viruses, for example Coxsackie B4, which are known to cause beta cell necrosis. The ease with which such viruses replicate in the beta cells may also be determined by the HLA phenotype. Other levels of genetic influence may relate to the severity of the autoimmune response and the potential for B-cell regeneration after virus-induced injury.

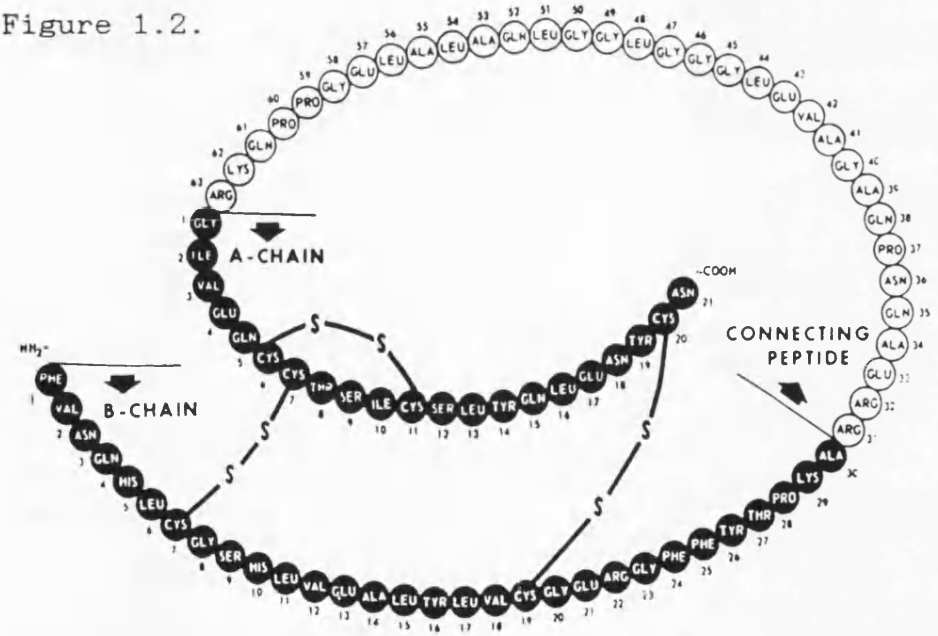
INSULIN

Type I diabetic patients are treated daily with heterologous insulin preparations of human, pork or beef origin.

Insulin is a small (5.75 Kd molecular weight) globular

protein composed of two polypeptide chains (A and B) that are linked by two disulphide bridges. Insulin is synthesised as a pro-insulin which consist of A and B chains linked by a connecting (C) peptide. (Structure of the pro-insulin is illustrated in Figure 1.2.). The C-peptide is cleaved by converting enzymes to yield the insulin molecule. The concentration of circulating C-peptide is often used to determine the natural history of beta cell function in diabetic patients (Block et. al., 1972) as exogenously administered insulin preparations do not contain the C-peptide.

Figure 1.2.



The structure of the porcine proinsulin molecule (after Shaw & Chance, 1968). The black circles indicate residues comprising the A and B chains of insulin; open circles indicate the connecting (or C) peptide. Residues 31 and 32, 62 and 63 are lost during cleavage of proinsulin to form free insulin and C-peptide.

The two main insulin analogues used to treat diabetic patients are pork and beef insulins. However, in the

United Kingdom, beef and pork insulins have been or are in the process of being phased out of clinical use as human insulin becomes more readily available. Beef insulin differs from human insulin by three amino acid exchange (Table 1.2.). Pork insulin differ only by one exchange. The C-peptides contain a much greater number of different residues compared to the human counterpart: ten for pork insulin and fourteen for the beef insulin molecule.

Table 1.2.

Species variation in amino acid sequence of human, pork and beef insulins (Reeves, 1980).

Species	A chain 8	A chain 10	B chain 30
Human	Threonine	Iso-leucine	Threonine
Pig	Threonine	Iso-leucine	Alanine
Ox	Alanine	Valine	Alanine

Insulin was first isolated in 1921 by Frederick G. Banting, Charles H. Best and others. It was used to treat patients with diabetes in the following year. Traditionally, insulin preparations had been purified by sequential recrystallization, but further examination of recrystallized material by gel chromatography in the late 1960's revealed the presence of contaminants which could be separated into three different fractions: (1)

contained high molecular weight impurities, (2) contained proinsulin, intermediatory insulin and insulin dimer and (3) contained arginine insulin, ethylester insulin and insulin monomer deaminated to a variable degree (Steiner et. al.,1968; Schlichtkrull et. al., 1972, reviewed by Reeves G.W., 1980). These contaminants were highly immunogenic and patients' sera contained high antibody titers to these antigens. Preparations obtained by removal of the (1) and (2) components were referred to as `single peak` (sp). These `sp' insulin preparations were still capable of inducing an antibody response but the amount produced depended on the physical state of the insulin preparation used therapeutically (Andersen, 1973).

Uncontaminated insulin monomer were separated by anion exchange chromatography which lead to the development of monocomponent (mc) highly purified insulins of pork and beef origin. These were less immunogenic as contaminating substances such as pancreatic polypeptide, glucagon, and vasoactive intestinal peptide (vip) which can induce antigenic reactions and the development of lipo-atrophy, were removed (Bloom et. al., 1979).

Recrystallized beef insulin when dissolved in physiological media produced an acid solution with a short duration of action. Adjustment of pH with an acetate buffer to form neutral soluble insulin caused less discomfort at the injection site but several

injections per day were necessary for adequate control. Various modifications were made to prolong the metabolic effect. Hagedorn et. al., in 1936 discovered that combining of the acidic insulin molecule with the highly basic protamine derivative from fish sperm in the presence of a moderate concentration of zinc ion yielded a stable complex with prolonged duration of action. This became known as neutral protamine Hagedorn (NPH) or isophane insulin. Other workers showed that a further increase in zinc and protamine concentration extended the period of action upto 3 days (Scott and Fisher, 1936). Protamine consisted of 86 per cent arginine and was considered non-immunogenic although apparent hypersensitivity has been experienced by some patients (Reeves, 1980).

The discovery that the activity of insulin could be prolonged by combining with zinc alone (Hallas-MØller, et. al., 1952) lead to the introduction of insulin-zinc suspensions whose duration of action could be adjusted by varying the relative proportions of non-crystalline (ie. amophous) and crystalline material within the insulin-zinc suspension. These `lente´ preparations contained a much higher zinc concentration than the isophane or soluble preparations. Highly purified insulin preparations were developed which were comparable with the classical `neutral´ preparations. `Isophane´ and `lente´ preparations and a twice daily combination of soluble and isophane preparations provided the most

flexible regime for the majority of insulin dependent diabetic patients.

In the late 1970's insulin of recombinant DNA origin was first produced and was first tested in human subjects in 1980 (Keen, 1980). Shortly afterwards 'semisynthetic' human insulin became available. It is a product manufactured from pork pancreatic insulin by the trypsin catalysed removal of the B30 alanine and its replacement by threonine (Markussen, et. al., 1983).

Most recombinant DNA human insulin is made by inserting synthetic genes for the A and B chains into plasmids at a promotor gene site. For example, at β-galactosidase or tryptophan synthetase, and then into Escherichia coli K12 (Crea, et. al., 1978). Following fermentation the chimeric gene product is cleaved, purified and the chains combined to make active insulin (Miller and Baxter, 1981). Today, human insulin from recombinant DNA proinsulin is a major commercial source.

Complications associated with Diabetes and Insulin Therapy

The administration of insulin to IDDM patients results in both cellular and humoral responses which, in a minority of patients, may lead to immunological complications. Insulin resistance and insulin allergy in the form of the

immediate or delayed type are two of the more common side-effects of insulin therapy.

Insulin resistance

The daily insulin dose requirement of a pancreatectomized diabetic is 40-50 units (Reeves, 1980). Most patients achieve good control of their diabetes with around 20 to 60 units per day. Patients requiring more than 200 units in the absence of ketosis are diagnosed as showing "insulin resistance". Patients with insulin resistance show much higher levels of circulating antibody (Berson & Yallow, 1959). However, the relationship between the level of insulin antibody and insulin dose in patients requiring only moderate levels of insulin has been a matter of debate. For example, Asplin, et. al., (1978), failed to find a relationship between insulin antibody and daily insulin dose. Much of the discrepancy has been due to (a) variations in diabetic management and attainment of `control', (b) variations in insulin preparations used to treat the patients and (c) variations in the methodology used to detect the insulin antibody. Despite these shortcomings, there is a reasonably clear distinction between the antibody levels seen in resistant and non-resistant patients (Reeves, 1980). The majority of patients showing a high daily insulin requirement (for example, more than 120 units), possess high levels of IgG insulin antibody in their

serum, (Berson & Yallow, 1959).

Skip et. al., (1965) has reported an incidence of 0.1 per cent of diabetic patients studied by them to show insulin resistance. Of the 107 IDDM patients studied in the present investigation, three are known to show insulin resistance (ie. requires more than 200 units per day). Therefore, insulin resistance is not uncommon even with highly purified and human insulin therapy. Insulin resistance may occur transiently during infection or episodes of ketosis and also in association with neoplasia, liver disease, other endocrine disorders and rare syndromes such as lipo-atrophic diabetes. In some patients a deficiency of insulin receptors on cell membranes is reported and in others the production of antibodies reactive with the insulin receptor may also lead to insulin resistance (Reeves, 1980).

Insulin allergy.

Insulin allergy is reported to develop in some patients during the initial period of insulin therapy and is characterized by the development of transient urticarial wheals or red nodules. Acidic soluble insulin preparations are known to give rise to painful reactions at the injection site in some patients. In contrast, less complications are reported with the use of buffered neutral preparations, although faulty injection technique may cause discomfort with any kind of insulin

preparation. Most studies implicate antibody of class IgE in the acute allergic response (Witters, et. al., 1977). It has been suggested that some cases of insulin allergy may be due to the presence of zinc in long acting insulin preparations (Reeves, 1986).

Lipo-atrophy.

Lipo-atrophy at the site of insulin injection occurs in about 25 per cent of patients treated with conventional beef insulins and is more common in female than in male patients (Reeves, 1980). Insulin lipo-atrophy is a local form of immune complex disease in which insulin and contaminating proteins react with insulin antibody and complement to produce inflammatory destruction of subcutaneous tissue. Histological analysis of the lipo-atrophic areas shows mild inflammatory changes with a peri-vascular lymphocytic infiltrate. Patients treated from the onset with highly purified insulins do not usually show any signs of lipo-atrophy (Poulsen & Deckert, 1976).

Most patients treated with conventional insulins produce complexes composed of insulin and anti-insulin antibodies (Reeves, 1986). Whether these complexes are involved in the pathogenisis of diabetic complications is debatable. For example, levels of circulating immune complexes have been found to be increased in the sera of patients with diabetic retinopathy when compared with patients without

retinopathy matched for duration of diabetes (Irvine, et. al., 1987). Because the patients used by Irvine included many who never received insulin, it is clear that complexes containing antigens other than heterologous insulins are involved.

Other complications which may be a side effect of insulin therapy include atherosclerosis. Coronary heart disease was found to be more prevalent among diabetic women especially in those receiving insulin therapy (Garcia, et. al., 1974). Most insulin-dependent diabetic patients have increased levels of circulating insulin which can retain its metabolic activity and stimulate arterial smooth muscle cell proliferation as well as lipid synthesis within the arterial wall (Stout, 1979). Therefore, it is possible that insulin therapy in conjuction with insulin antibody production may have a role in exacerbating the underlying proneness of diabetic patients to develop atherosclerotic disease.

The above immunological complications are found in a minority of patients. However, most diabetic patients undergoing insulin therapy show cellular and antibody responses to insulin, which do not necessarily lead to any adverse effects.

In the following chapters the immune response to insulin in two groups of patients is examined. Group I consisted of IDDM patients on human insulin therapy for more than

six months. Group II comprised of IDDM patients on beef insulin who later were transferred to human insulin therapy.

The main objectives of this study were to investigate the *in vitro* cellular immune responses to pork, beef and human insulins in order to assess the level and specificity of response to the three types of insulin, and to examine the possible associations between the cellular immune response to insulin and the clinical background of the patients.

Group II patients, were used to investigate the effects of transferring from beef to human insulin therapy on the *in vitro* cellular immune response to human, pork and beef insulins. The effects of changing from beef to human insulin therapy on the level of their anti-insulin antibody titer were also examined.

An Insulin-specific, Peripheral Blood Mononuclear Cell (PBMC) proliferation assay was employed as a measure of cellular immune response with emphasis on suppressor cell activity. The humoral immune responses to pork, beef and human insulins were determined using the Enzyme Linked Immunosobent Assay (ELISA) with emphasis on serum anti-insulin IgG concentration and anti-insulin IgG subclass distribution.

The presence of other Ig Isotypes (namely, IgE and IgM), in the patients' sera, were also determined.

CHAPTER II

2. *CELLULAR IMMUNE RESPONSE TO INSULIN*

2.1. INTRODUCTION

Treatment of type I diabetic patients with either heterologous insulin (pork insulin (PI), beef insulin (BI)) or autologous insulin (human insulin (HI)) induces the production of anti-insulin antibodies in the serum of most patients (Pickup, 1986). A cellular immune response to insulin, in the form of insulin-specific lymphocyte transformation, is also observed in some IDDM patients (Mann, et. al., 1983; Nell, et. al., 1983). In a minority of these patients immunological complications may result even with the use of highly purified and human insulin preparations (Reeves, 1985; Pickup, 1986). Immunological complications associated with insulin therapy are discussed in detail in Chapter I - `Introduction´.

Most complications related to insulin therapy, implicate insulin antibody as the culprit. However, antibodies are produced by transformed B-lymphocytes as the final step in the expression of the humoral immune responses. The initial step is the interaction of the insulin molecule with antigen presenting cells and T-lymphocytes with subsequent blast transformation of the T-lymphocytes to

cells capable of promoting the transformation of the B-lymphocytes to effector cells of the plasma cell series. Therefore, in order to understand the humoral immune response to insulin, it is necessary to investigate the cellular response. Moreover, *in vitro* assays of T-cell stimulation may yield more subtle information on insulin reactivity than antibody studies.

Compared to the numerous investigations on the antibody response to insulin, few workers have concentrated on the the cellular immune response to therapeutically administered insulin and its relationship to the clinical background of patients.

T-cell proliferation assays are commonly used as a measure of cellular immune response to insulin in type I diabetic patients. In the past, most of these studies have implicated the amino acid differences between autologous insulin and immunizing insulins as the critical epitopes on the insulin molecule recognized by T-cells (Mann, et. al., 1983; Scheinin, et. al., 1983). Recent investigations, however, show that recognition of amino acid exchanges is not the only factor determining insulin immunity. Results from experiments using T-cell clones (Parker & Reeves, 1989) demonstrate that the potential immunogenic epitopes recognized by T-cells are more numerous and complex than anticipated from results with inbred animals.

T-cell populations obtained from type I diabetic patients

react with epitopes present on foreign and self insulin (Nell, et. al., 1983; Nell, et. al., 1985; MacCuish, et. al., 1975; Page-Faulk, et. al., 1975; Naquet, et. al., 1988). Some of these epitopes may be shared between different species of insulin (for example, BI, PI, Rabbit Insulin (RI) and HI), due to the high degree of sequence homology. T-cell reactivity to such "common epitope(s)" present on HI is thought to lead to autoimmunity (Nell, et. al., 1985).

Although, it's structure was not precisely identified, one such epitope was reported to consist of a complex determinant formed by the association of the A-chain loop with the N-terminal residues of the B-chain (Nell, et. al., 1985). Recently, it has been shown that autoreactive T-cells recognize two conformational epitopes of human insulin formed by interactions between A-chain and B-chain residues (Naquet, et. al., 1988). One epitope is associated with the A-chain loop and is present in the A1-A14/B1-B16 peptide, and the other in the A16-A21/B10-B25 peptide. These two epitopes are present in amphipathic α-helical regions of the insulin molecule.

Studies with inbred animals show that the immune response to epitopes on the insulin molecule is controlled at the cellular level by the major histocompatibility complex (MHC)-linked immune response genes (Erb, 1980; Glimcher, et. al., 1983; Jensen & Kapp, 1985; Rosenwasser, et. al., 1979). Recently, several studies (Mann, et. al.,

1983; Scheinin et. al., 1983; Sklenar, et. al., 1982; Miller, et. al., 1987; Naquet, et al., 1988; and Parker & Reeves, 1989) have reported an association between class II MHC encoded gene products and the response to therapeutic insulin components in diabetic patients and therefore, support the concept that similar immune response genes are present in the outbred human population.

Individuals whose lymphocytes respond to either BI or both BI and PI are reported to express an increased frequency with respect to the normal population of the HLA-DR4 and HLA-DR3 antigens respectively. Furthermore, HLA-DR (DR3, DR4, and DR5) and HLA-DQ (DQw2/DQw3) antigens can restrict these T-cell responses to human epitopes. It is thought that responses to HI epitopes is restricted by α- and β-chains of DQw2 and DQw3 molecules which produce hybrid molecules in DR3/4 heterozygotes (Naquet, et. al., 1988).

Miller, et. al., (1987), observed that human T-lymphocyte responses to mamalian insulins are polyclonal and include T-cells with auto-immunity for human insulin. They report that different T-cells recognize the same epitopes. For example, A-loop of beef insulin, in association with either DR1 or DRw6. The response pattern of T-cell lines (TCL) that cross-react with pork or human insulin suggest that some T-cells have slightly different fine specificity for the A-chain loop

region where beef insulin differs from pork and human (A8 and A10). One TCL (P4/1) derived by pork insulin stimulation reacts with both beef and pork but not human insulin. Thus, the B-chain terminus (B30) where beef and pork insulin differ from human insulin may be an important component in the epitope recognized by this clone. This residue (B30) is topographically associated with the A-chain loop on the surface of the insulin molecule (Blundell, et. al., 1972), and may form a conformational determinant recognized by these T-cells.

It is thought that A-loop residues interact with Ia and display other portions of the molecule for recognition by T-cells (Miller, et. al., 1987). This is further substantiated by Glimcher, et. al., 1983) who found that the immunogenic moiety recognized by T-cell hybridomas is formed by the amino acid sequence of the B-chain (possibly involving amino acid B3) and by the interaction of the B-chain with the A-chain loop region. The preferred antigenic moiety recognized by these T-cell hybrids depends upon tertiary conformation, thus, although the group II T-hybrids can respond to isolated B-chain, they are triggered more easily by native molecule.

Insulin-specific suppressor T-cells

Suppressor cell activity in IDDM patients is reported to be depressed at the time of diagnosis and normal in the

remission period (Buschard, et. al., 1982). Horowitz et. al., (1977) found decreased suppressor T-cell function in 6 of 9 patients with IDDM; not all were newly diagnosed. In comparison, all control subjects showed normal suppressor cell activity. The decrease in the number of functional suppressor cells is thought to contribute to the acceleration of the auto-immune process of type I diabetes. Indeed, many physicians in recent years have employed immuno-suppressive drugs such as cyclosporin A in order to retard the autoimmune process in newly diagnosed diabetics (Herold and Rubenstein, 1988).

Although the present study is not necessarily concerned with the auto-immune aspects of IDDM, it would be of great interest to determine whether a deficient suppressor cell activity exist in established diabetic patients which may lead to all sorts of immunological complications. As most IDDM patients retain some beta cell function, lack of immunoregulation by suppressor cells may contribute to the greater loss of beta-cell secretory capacity, which in turn may influence diabetic control and insulin dose requirement.

Most studies investigating the possible role of metabolic control on suppressor cell activity have used assays which were non-specific, eg., the use of Concanavalin A to stimulate suppressor T-cells. However, it is well established that suppressor cells are antigen-specific.

ie., they recognize and distinguish between antigenic moieties (Baskin & Rosenthal, 1980; Kontainen, 1982; Jensen & Kapp, 1985). Therefore, lack of any relationship between suppressor cell activity and patients' clinical background which has been reported in the past (Buschard, et. al., 1982) may be due to inappropriate methodology. That insulin-specific suppressor cells exist, there is little doubt. How these suppressor cells function is discussed below.

In some inbred animals and diabetic patients there is no apparent antibody response to the immunizing insulin. For example, mice bearing the H-2^b haplotype, develop insulin-specific antibody responses after immunization with beef and sheep insulin, but not pork or autologous insulin. H-2^b mice injected with pork insulin develop memory helper T-cells that support secondary responses to beef, but not pork insulin in adoptive recipients and *in vitro*. Pork insulin stimulates a heteroclitic pattern in H-2^b mice since the primed T-cells support secondary responses to beef insulin but not to the immunizing antigen.

Non-responsiveness is not due to a lack of insulin specific B-cells but to a lack of functional helper T-cells. ie., non-immunogenic variants of the insulin molecule prime memory helper T-cells but are unable to trigger the primed T-cells to co-operate with B-cells. This is because pork insulin also primes dominant pork

insulin-specific suppressor T-cells in H-2^b mice (Jensen & Kapp, 1985). The heteroclitic response pattern observed can be explained by the differing antigenic fine specificities of pork insulin-primed helper and suppressor T-cells.

Whereas the helper T-cell is cross-reactive for pork and beef insulins, the suppressor T-cells recognize pork but not beef insulin. The net effect, in the presence of both regulatory subpopulations is helper activity for secondary responses to beef but not pork insulin. This implies that suppressor and helper T-cells recognize two different epitopes on the same insulin molecule or, at least, that responsiveness to insulin and insulin suppression are under different genetic control (Kontianien, 1982).

Jensen and Kapp (1985) suggest that, in mice at least, suppressor T-cells recognize determinants on the A-chain loop, since that is the only site where the primary amino acid sequence differs between pork and beef insulins. This conclusion is verified by the observation that H-2^b mice immunized with pork or rat insulin (rat insulin is homologous to mouse insulin and the A-chain loop of pork and mouse is identical) contain primed helper T-cells and dominant suppressor T-cells that recognize mouse insulin.

Parker and Reeves (1989), using an *in vitro* human

lymphocyte priming assay, also came to the conclusion that the epitope recognized by human insulin-specific suppressor cells reside in the A-chain. They found that secondary challenge with an insulin which is more foreign than that used for priming can elicit a greater response at the end of secondary culture *in vitro*. This is most apparent when cultures primed with pork insulin are challenged with beef insulin. It was postulated that the *in vitro* response to pork insulin is regulated by cells activated by suppressor epitope(s) present on both human and pork insulins. If such an epitope is located within the A-chain loop (ie., residue A8-A10) then this would explain the enhanced response when challenged with insulin in which these residues are altered (eg., beef and ovine insulins) because this would interfere with the suppressive effect.

Suppressor T-cells are thought to mediate their function via soluble mediator molecules (suppressor factors) (Kontiainen, 1982). These suppressor factors are not always genetically restricted and may act across species barriers. For example, antigen specific suppressor factors of monkey or human origin can be tested on mouse cells (Lamb et. al., 1979). Using antigens under Ir gene control it has also been demonstrated that both responder and non-responder strains produce these suppressor factors. (Baskin and Rosenthal, 1980).

Insulin-specific suppressor factors resemble other human

secreted antigen specific suppressor factors in that they carry the factor constant (function related) and DR determinants, and in acting across a species barrier (Kontiainen, 1982). However, whether the suppressor factor is analogue specific is debatable. For example, both beef and pork insulins induce suppressor factors that abolish pork insulin specific T-helper cell activity (these same factors do not affect keyhole limpet haemocyanin (KHL) specific T-helper cells). This lack of insulin analogue specificity was not observed when immunoabsorbtions were performed; all detectable suppressor activity in pork insulin induced suppressor factors was absorbed out when pork insulin immunoabsorbents were used but not when beef insulin immunoabsorbents were used. Beef insulin suppressor factor activity was equally specific for beef insulin immunoabsorbents (Kontiainen, 1982).

This discrepancy between lack of analogue specificity at the functional level and specificity with immunoabsorbance, may be due to several reasons: contamination by either analogue in culture media; alternatively, multiple, partially overlapping suppressor cell inducing determinants exist which are probably influenced by the physicochemical forms of the insulin molecule. ie., insulin is mainly monomeric at concentrations < 1 $ug.ml^{-1}$ and mainly hexameric at doses > 30 $ug.ml^{-1}$. The determinants recognized may well be located in the B-chain as beef insulin-specific

suppressor factor of H-2^b mice (with T-helper cells recognizing A-chain determinants) was not absorbed by beef A-chain (Kontiainen, 1982).

Thus insulin antigen seems to trigger suppressor pathways in addition to insulin antibody, delayed hypersensitivity or allergic reactions. The suppressor pathways probably act to diminish unwanted reactions towards this self antigen ie., act as specific immunoregulation.

In contrast to the numerous investigations into the antibody specific immune response to insulin, the possible role of T-suppressor cells in the immunoregulation of insulin therapy in type I diabetics has been rather neglected.

Since human insulin has come into clinical use quite recently (many of the patients used in this study were transferred from pork and beef insulins to human insulin during the last three years), this study takes the opportunity to investigate the cellular immune response to this autologous insulin in greater detail with a larger number of patients than previously reported (Naquet, et al., 1988).

Furthermore, insulin-specific suppressor cell activity in diabetic patients has not been investigated in detail, although, Naquet, et. al., (1988) using a different methodology to the one used here has made a preliminary study which involved a limited number of patients (four

families). In this chapter the clinical importance of insulin-specific suppressor T-cells (OKT8 +ve), and their possible role in the regulation of cellular immune response to insulin is discussed.

2.2. PATIENTS, MATERIALS & REAGENTS

2.2.1. *PATIENTS*

Type I diabetic patients, who were otherwise healthy out-patients with well controlled diabetes, were divided into 2 groups on the basis of their insulin therapy. The third group comprised of healthy non-diabetic volunteers whose mean age, sex, and Body Mass Index (BMI) were matched with those of the diabetic patients, (see table 2.1.). All diabetic subjects were caucasians.

Group I: Type I diabetic patients on human insulin therapy for more than six months.

Group II: Type I diabetic patients on highly purified beef insulin (Neusulin & Neuphane) therapy who later were transferred to human insulin (Human Velosulin (emp) & Insulatard (emp)). - Blood and serum samples were taken while the patients were on beef insulin, 3-8 months after they were transferred to human insulin, and 12 months later.

Table 2.1.

Group III: Healthy non-diabetic control subjects.

	Group I	Group II	Group III
N	63	31	22
Mean Age (years) (range)	36.2 (16-54)	41.6 (21-56)	35.7 (19-52)
Sex	35 male 28 female	21 male 10 female	12 male 11 female
Mean Body Weight (Kg) (range)	70.96 (50-106)	Not known	64.98 (54-75)
HbA1 (range)	11.7% (7.7-16%)	Not known	7.5% (5-9.5%)
Duration of Insulin Therapy in years (range)	12.9 (1-35)	Not known	- -

N=number of patients.

PATIENTS' INSULIN THERAPY:

Group I: Most patients were administered one or two of the following insulin preparations twice daily:

Human Actrapid (Novo); short acting, highly purified synthetic human human neutral insulin (emp).

Human Monotard (Novo); intermediate-acting, highly purified synthetic human insulin zinc suspension (emp).

Human Ultratard (Novo); long-acting, insulin zinc suspension (crystalline) human insulin (emp).

Human Insulatard (Nordisk & Wellcome); intermediate

acting highly purified human isophane insulin (emp).

Human Mixatard 30/70 (Nordisk & Wellcome); Biphasic prep. Highly purified human insulin (emp), 30% neutral soluble insulin and 70% isophane insulin.

Humulin Soluble (Eli Lilly); short-acting human soluble insulin (prb).

Humulin Isophane (Eli Lilly);intermediate-acting human, isophane insulin (prb).

Group II: 1st sample - All patients had been receiving twice daily preparations of the following insulin types: Neusulin (Wellcome); short-acting, highly purified beef insulin. Neuphane (Wellcome); intermediate acting, highly purified beef insulin. 2nd & 3rd samples were taken when the patients were undergoing the following insulin therapy:

Human Velosulin (Nordisk and Wellcome); short-acting, highly purified neutral soluble human insulin (emp).

Human Insulatard (Nordisk & Wellcome); intermediate acting highly purified human isophane insulin (emp).

All insulin preparations were U100. emp = enzyme modification of pork insulin; prb = produced from proinsulin synthesised by bacteria using recombinant DNA technology.

2.2.2. *MATERIALS & REAGENTS*

(i) Preparation of culture medium : RPMI 1640 medium (Flow Laboratories, Ltd., England) was obtained as a x 10 strength media in 500 ml unit quantities. Antibiotic solution: Penicillin (5,000 $ug.ml^{-1}$) and Streptomicine (5,000 $ug.ml^{-1}$) sterile solution (Flow Labs. Ltd.), was stored at -20°C. 200mM, sterile L-Glutamine solution (Flow Labs., Ltd.), was stored at -20°C. Sodium bicarbonate was also obtained as a sterile 7.5 % w/v NaHCO3 (Flow Labs., Ltd). Foetal Bovine Serum (FCS) (Gibco Ltd., England). The FCS came heat inactivated at 56°C for 30 minutes. It was stored at -20°C. Human AB Serum was obtained from Southmead Hospital, Bristol.

Table 2.2.

Human AB serum and additives were aliquoted into sterile 30 ml Sterilin Universal tubes and stored at -20°C until further use. For every 100mls of culture medium the following were added:

Component	Volume added	Final concentration
200 mM L-Glutamine	1 ml	2mM
5000 $u.ml^{-1}$ Benzyl Penicillin }		50 $u.ml^{-1}$
5000 $u.ml^{-1}$ Streptomycin }	1 ml	50 $ug.ml^{-1}$
7.5 % w/v Sodium bircarbonate	2.67 mls	23.8 mM
Human AB Serum (or FCS)	10 mls	10%

Preparation of single strength medium form x10 concentrations:- Deionized distilled (d.d) water was sterilized by autoclaving at 121°C for at least 20

minutes. 100 mls culture media was prepared as follows: To each bottle of 75.33 mls of d.d. water, 10 mls of x 10 RPMI 1640 medium and 1 tube of Human AB serum and additives were added. The pH of the culture medium was found to be between pH 7.2 and 7.4.

(ii) Insulin solutions : Crystalline zinc beef, pork and human (biosynthetic-recombinant DNA) insulins were kind donations of Eli Lilly Co., Lilly Corporate Center, Indianapolis, IN., U.S.A.

	Type of Insulin		
	Beef	Pork	Human
Potency	26.6 U.mg^{-1}	26.8 U mg^{-1}	26.3 U mg^{-1}
Proinsulin	<0.001%	<0.0001%	-
Glucagon	<0.001%	<0.0001%	-
Zinc	<0.7%	<0.9%	0.4%
Water	9%	7%	8.3%
Mol. Wt.	ND*	5778 daltons	5808 daltons

ND = not determined.*

5 mgs ml^{-1} of the crystalline insulin was made up in dilute acid (5 x 10^{-3} M HCl, pH 2.5), filter sterilized and stored at 4°C (for upto one week) in 200 ul aliquotes. For the proliferation assay, aliquots of these stock solutions were diluted further with RPMI 1640 medium supplemented with 10 % AB serum and additives.

(iii) Phytohemagglutinin (PHA)-Purified, phaseolus spp. (5ml, dried) (Wellcome Foundation Ltd., UK). The content was reconstituted with 1 ml of sterile d.d. water to give a concentration of 10 mg ml^{-1} and stored at 4°C. This stock solution was diluted 1:100 with RPMI 1640

medium (containing 10 % Human AB serum and additives) to a concentration of 100 ug ml^{-1}. The diluted PHA was filter sterilized using 0.2um Milipore filter and aliquoted into 100 ul volumes to be stored at -20°C.

(iv) Lymphocyte separation medium (density = 1.077 $g.ml^{-1}$; 500ml bottle) (Flow Labs., England).

(v) Phosphate Buffered Saline (PBS) (Dulbecco `A`) pH 7.3, was obtained as tablets (Oxoid Ltd., England). Each tablet was dissolved in 100 mls of d.d. water and sterilized by autoclaving. The sterile PBS was stored at 4°C.

(vi) Methyl [^{3}H]- Thymidine (925 G.Bq m mol^{-1}; 37M.Bq,1 M.Ci) (from Amersham, UK). 1Mci ml^{-1}, was diluted 1:100 with RPMI 1640 media containing 10% human Ab serum and additives to give a concentration of 10 uCi ml^{-1}.

(vii) LKB Optiphase `Safe` (FSA Laboratory Supplies, Loughbrough, Leics, England).

(viii) PBS-Azide (PBSA), PBS with 0.2 % sodium azide.

(ix) 5 x 10^{-2} M Tris buffer pH 9.5 (Sigma Chem., Ltd., England) was made up in d.d. water and sterilized by autoclaving.

(x) 40 % Formaldehyde.

(xi) White Cell Staining Fluid : 3 % Acetic acid and 0.1 % Methylene Blue made up in d.d. water.

(xii) Mounting Fluid (DABCO) for Fluorescence microscopy was made up of the following : 90 mls of Glycerol, 10 mls of PBS (150mM Sodium Chloride; 150mM Sodium Phosphate, pH 7.2) and DABCO (1,4-Diazabicyclo-[2,2,2,] octane; Triethylene diamine) from Sigma Chem. Co., Dorset, U.K.

(xiii) Fluorescein conjugated anti-immunoglobulin (Rabbit anti-mouse/FITC) (Nordic Immunological laboratories, Madienhead, Berkshire) - the content of the tube was reconstituted with 2 mls of cold sterile water, spun down to remove insoluble particles and divided into 100 ul aliquotes and stored in the dark at -20°C.

(xiv) Affinity purified
Sheep anti-Mouse IgG (5 mg) (Nordic Immunological laboratories, Madienhead, Berkshire).

(xv) Monoclonal antibodies (raised in mice) : OKT4 and OKT8 supernatants were kindly donated by Dr. K. Moore and Mr. A.M. Nesbitt of TENOVUS Laboratories, Southampton General Hospital, Southampton.

(xvi) Sterile 96-well, polystyrene round-bottomed microtiter plates (Corning Glass Works, Corning, N.Y. 14831).

(xvii) Individually packed 0.2 um Nitrocellulose filters (from Milipore) were used to sterilize solutions which could not be autoclaved.

(xviii) Siliconized pasteur pipettes : pasteur pipettes were coated by twice drawing up dimethylsilane solution (BDH Chemicals., UK) into them. The pipettes were allowed to air dry and then immersed in a large volume of freshly collected distilled water for a minimum of 30 minutes. The siliconized pipettes were dried in a warm air oven, sealed in autoclave bags and sterilized by autoclaving.

(xix) ILACON Titer-tek cell Harvester and filter paper (Flow Labs., Skatron, Lier, Norway).

(xx) 100 x 15 mm polystyrene bacteriological petri dishes (Sterilin, Feltham, England).

(xxi) 25 ml Universals and 10 ml conical tubes (Sterilin, Feltham, England.

(xxii) Microscope slide and coverslip.

(xxiii) Improved Neubauer Hemocytometer.

(xxiv) Beckman TJ-6 centrifuge.

(xxv) Fluorescent Microscope.

(xxvi) Phase-Contrast Microscope.

(xxvii) Class 2 Flow cabinet (Flow Labs., England).

(xxviii) CO_2 Humid Incubator.

(xxix) LKB Wallac 1215 Reckbeta Liquid Scintillation Counter

2.3. METHODS

2.3.1. Separation of Peripheral Blood Mononuclear Cells (Boyum, 1968)

Blood samples from diabetic patients were taken 1 to 3 hours after their first insulin dose of the day. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by the Ficoll-Hypaque gradient separation technique using modification of Boyum's gradient centrifugation method (Boyum, 1968).

A 1:1 mixture of blood and PBS was layered over 5 mls of lymphocyte separation medium (specific density of 1.077 $g.ml^{-1}$) and centrifuged at 400 x g for 30 minutes in a Beckman TJ-6 centrifuge. Cells at the interface were harvested and washed three times with PBS containing 5 % AB serum before being suspended at a density of 10^6 cells ml^{-1} in RPMI 1640 medium containing 10 % Human AB serum and additives.

The cells were checked for viability using trypn blue stain and examined under phase contrast microscope. For all suspensions viability was found to be > 95 per cent.

From 20 mls of blood, between 1.55×10^7 and 3.65×10^7 cells were obtained, depending upon the individual subject.

2.3.2. "Panning"-Fractionation of T-Cell Subpopulation.

(Wysocki & Sato, 1978; Moore & Nesbitt, 1986)

Antibody molecules adsorb on to polystyrene surfaces and bind antigen (Catt & Tregear, 1967). Therefore, it is possible to coat polystyrene dishes with antibody specific for cell surface antigens and permit cells to bind to such dishes. The fractionated cells are recovered in high yield with low levels of contamination by other cell types. The recovered cells retain immunogenic functions (Wysocki & Neverley, 1978).

T-cells defined by anti-leu2 and OKT8 antibodies mediate most cytotoxic and suppressor functions. T-cells defined by anti-leu 3 and OKT4 antibodies mediate most helper/inducer functions (Damle et. al., 1983). On the basis of this phenotypic classification OKT8 monoclonal antibody was used to "pann-out" CD8 (suppressor/cytotoxic) cells in order to investigate insulin specific suppressor cell activity in an *in vitro* cellular proliferation assay.

2.3.2.(a) Direct "Panning"

2 ml of OKT8 supernatant was diluted with 3 mls of 50mM Tris buffer (pH 9.5) and poured into each polystyrene petri dish. Following 90 minutes incubation at room

temperature, with occasional swirling, the buffer-antibody solution was decanted and the petri dish was washed four times with PBS containing 1 % Human AB serum. The AB serum blocks any remaining binding sites on the plate.

1-2 x 10^7 peripheral blood mononuclear cells were resuspended in 5 mls of PBS containing 5% Human AB serum and gently poured into each antibody coated petri dish. The plates were incubated on a level surface at 4°C for 70 minutes, with gentle swirling every 10-15 minutes. Following incubation, the non-adherent cells were transferred to a second antibody-coated petri dish for a further 70 minutes incubation period.

The adherent cells remaining on the first petri dish were gently washed four times to remove any non-adherent cells. Cells bound to the petri dish were recovered by vigorous pipetting and washing with PBS containing 5 % AB serum.

The above procedure was repeated with the second petri dish and the adherent cells were pooled. The non-adherent cells were also pooled into a separate tube. This `double panning` technique gave rise to two cell populations : non-adherent, OKT8 -ve cells; and adherent, OKT8 +ve cells.

2.3.2.(b) Indirect "Panning"

Polystyrene petri dishes were coated by adding affinity purified sheep anti-mouse IgG (25 ug per plate) in 5 mls of 0.05 M Tris buffer (pH 9.5) and incubated at room temperature for 90 minutes. Following incubation the plates were washed 3 times with PBS (containing 1 % serum) to remove excess antibody and incubated with PBS containing 1 % AB serum for 15 minutes at room temperature. The plates were washed twice and stored at -20°C to be used for upto 3 months or at 4°C overnight (O/N).

Peripheral blood mononuclear cells (1 x 10^7) were incubated with 500 ul of OKT8 culture supernatant at 4°C for 30 minutes. The cells were washed twice with PBS-5 % AB serum. The cells were resuspended in 5 mls of PBS-5 % AB serum and added to the sheep anti-mouse IgG coated petri dish.

After 60 minutes at room temperature (with gentle swirling every 10-15 minutes) the non-adherent cells, depleted of CD8+ cells, were harvested. The petri dishes were gently washed four times with PBS-1 % AB serum and the adherent cells (OKT8 +ve) were recovered by vigorous pipetting and washing.

2.3.3. Phenotypic Characterization

Indirect immunofluorescence was used to assess the purity of the separated cell populations. 1 x 10^6 cells were coated with the primary unlabelled monoclonal antibody by incubating the cells with 50 ul of OKT8 or OKT4 supernants at 4°C for 15 minutes. This was followed by four washes with cold PBS containing 0.2% sodium azide (PBSA, the azide prevents capping). The cells were resuspended in 100 ul of 1 in 20 diluted rabbit anti-mouse IgG/FITC (Nordic, The Netherlands) and incubated at 4°C for 15 minutes. Excess fluorescein conjugated anti-mouse IgG was removed by four more washes with PBSA. The final wash was followed by resuspension of the cells with 20 ul of PBSA, 5 ul of which was mounted on to microscope slides. The cells were fixed and dried: the slides were placed into a sealed container with 40 % formaldehyde for 15 minutes and allowed to dry O/N at 4°C in a damp atmosphere. The cells were finally covered with a drop of DABCO-permanent mounting fluid, covered with a cover slip and sealed with nail polish.

The cells were examined using a fluorescence microscope under x20 and x100 objectives. The results in Table 2.3. gives the mean percentage of positively labeled cells + standard deviation of 153 observations. Contaminating cells (not-fluorescent) include B-lymphocytes, monocytes and granulocytes.

Table 2.3.

Phenotypic Characterization :

Primary Label	Cell Population Unfractionated	OKT8 -ve	OKT8 +ve
OKT4	56.0% + 7	62.7% + 5	3.0% + 2
OKT8	27.4% + 8	2.3% + 4	74.1% + 4
Total	83.4%	65%	77.1%
Contaminating cells	16.6%	35%	22.9%

In order to validate the "panning" technique Flow Cytofluorography was employed using a Coulter EPICS Profile Analyzer, with kind permission of Mr. C. Axton of Lilly Research Centre Ltd. The Coulter EPICS profile analyzer is an optical/electronic device that measure cell size and detects the presence of cell-bound fluorochrome-labeled antibodies.

Figure 2.1. shows three such cytofluorographs:

FS refers to `Forward Scatter` signal. It is a measure of cell size. As dead cells give rise to a smaller forward scatter signal than live cells, this parameter is particularly useful in discriminating between viable and non-viable cells. It is also able to distinguish between nucleated cells and mammalian erythrocytes.

LSS gives the 90° Light Scatter (or Side Scatter) value. It is a measure of the heterogeneity of cell structure. Thus cells with large numbers of cytoplasmic granules or other organelles scatter more light than erythrocytes or

lymphocytes. Simultaneous measurement of FS and LSS allows identification of lymphocytes, monocytes and granulocytes in peripheral blood.

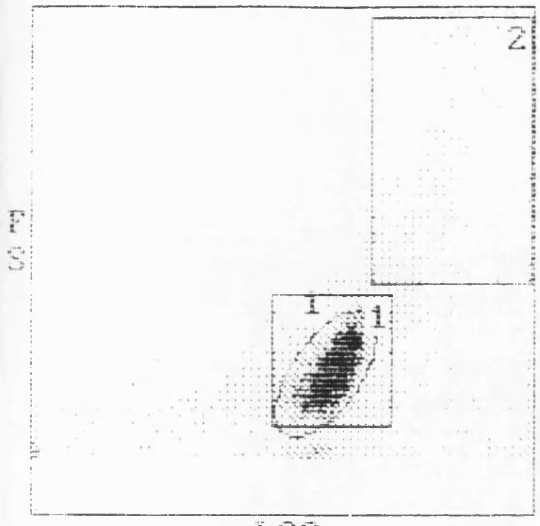
Figure 2.1.(a) shows the `X-Y` dot display of scatter signals generated by Peripheral Blood Mononuclear Cells (PBMC) separated using the protocol outlined in section 2.3.1. 80.9% of the PBMC was found to be Lymphocytes (Square 1), and 6% was found to be granulocytes and monocytes (Square 2).

Figure 2.1.(b) : 100 ul of 1 $x10^6$ unfractionated cells was labeled with 10 ul of T4-Phycoerythrine (LFL2) and 10 ul of T8-Fluorescein isothiocyanate (LFL1). Following 15 minutes incubation the cells were analysed on the Coulter EPICS profile analyzer. The results show that 40.9% of the unfractionated cells are T4 positive (LFL2) and 19.3% are T8 positive (LFL1).

Figure 2.1. : gives the cytometric profile of OKT8-ve cell population (prepared by "panning" - see section 2.3.2.(a)). The cells were labeled with T4-Phycoerythrine (LFL2) and T8-FITC (LFL1). The results show that 41.7% of the cells are T4 positive and only 1.7% are T8 positive. This indicates that the OKT8-ve cell population contains few contaminating T8+ve cells, although there appears to be other contaminating cells which are not lymphocytes. Since, the aim of the "panning" technique is to deplete the OKT8-ve cell population of $T8^{+ve}$ (suppressor/cytotoxic) cells only,

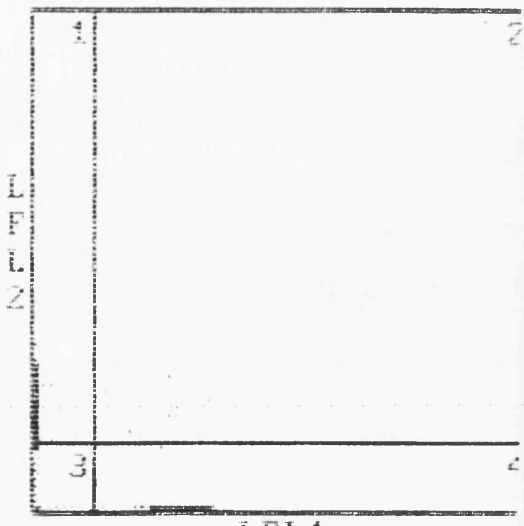
Figure 2.1.(a).

EPICS® Profile Analyzer
COULTER CYTOMETRY TEST RESULTS



LSS

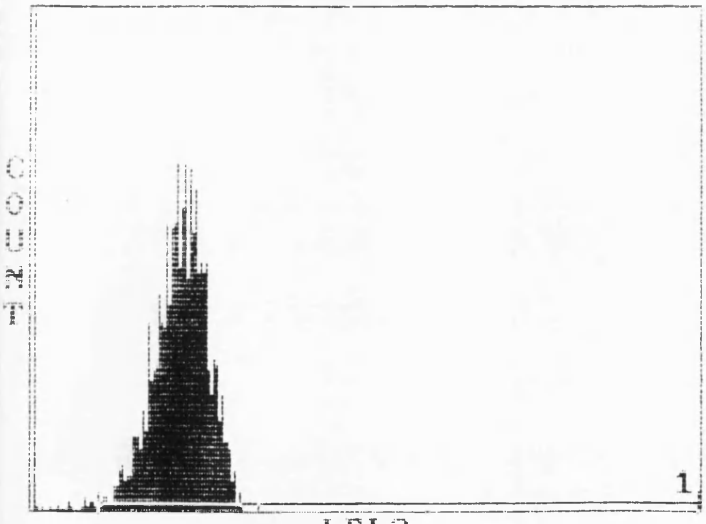
		MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
1	X	26.09	146.6	5251	80.9	57.62	1.37	7.03
	Y	11	27			17.7	3.4	20.7
2	X	106.2	1023.	366	5.6	223.4	1.6	4.94
	Y	29	62			37.2	7.3	11.6



LFL1

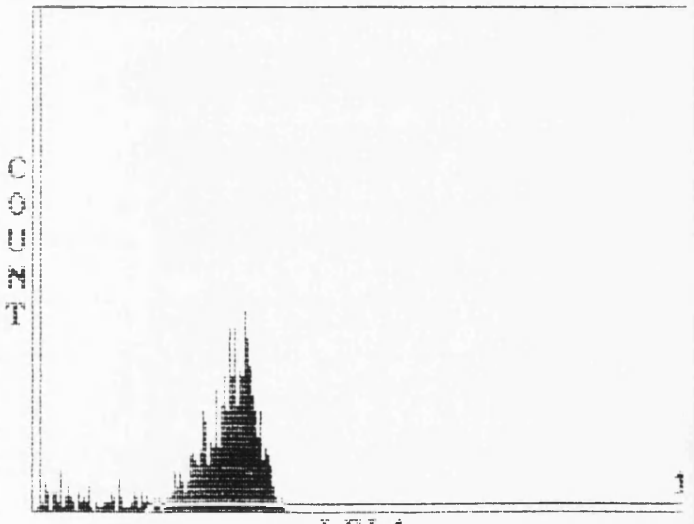
		MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPC
1	X	1.024	2.427	2111	42.2	1.024	1.034	
	Y	2.705	1023.			4.364	1.304	16.
2	X	2.428	1023.	6	0.1	5.656	1.615	3.7
	Y	2.705	1023.			4.397	1.301	4.2
3	X	1.024	2.427	2015	40.3	1.040	1.118	
	Y	1.024	2.703			1.062	1.205	
4	X	2.428	1023.	869	17.4	7.193	1.417	9.1
	Y	1.024	2.703			1.025	1.054	

Figure 2.1.(b).



LFL2

	MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
1	2.010	1023.	1483	40.9	4.557	1.340	15.2



LFL1

	MIN	MAX	COUNT	PERCENT	MEAN	SD	% HP
1	3.357	1023.	702	19.3	7.754	1.340	5.

#23
4:19 p.m
9-Jun-8
HUMAN LYMPH
T4RD1T4FI1

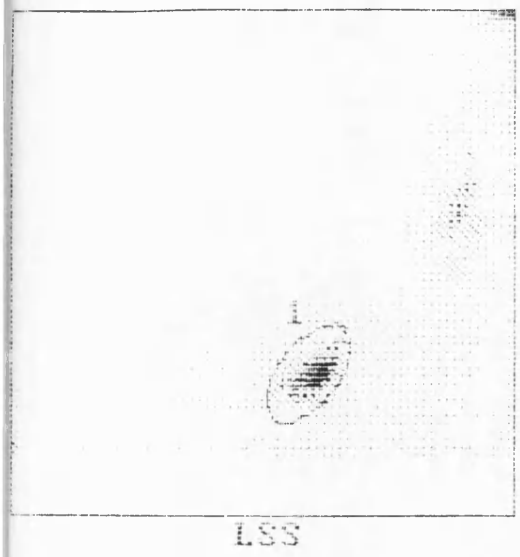
UNFRACTIONATE

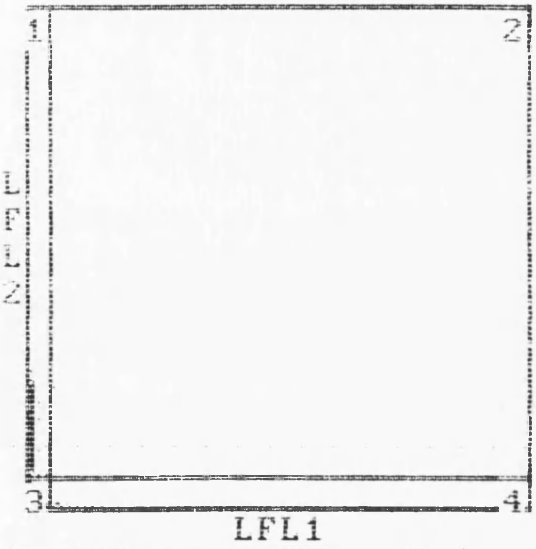
EPICS is a registered trademark of Coulter Corporation.

Figure 2.2.

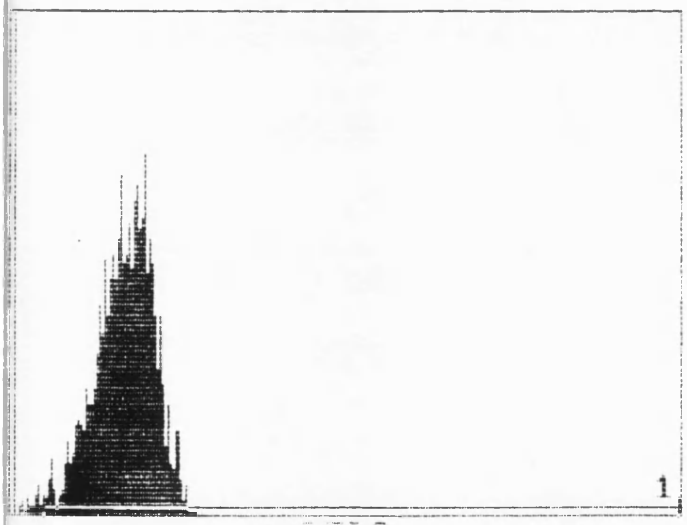
EPICS(R) Profile Analyzer

COULTER CYTOMETRY TEST RESULTS

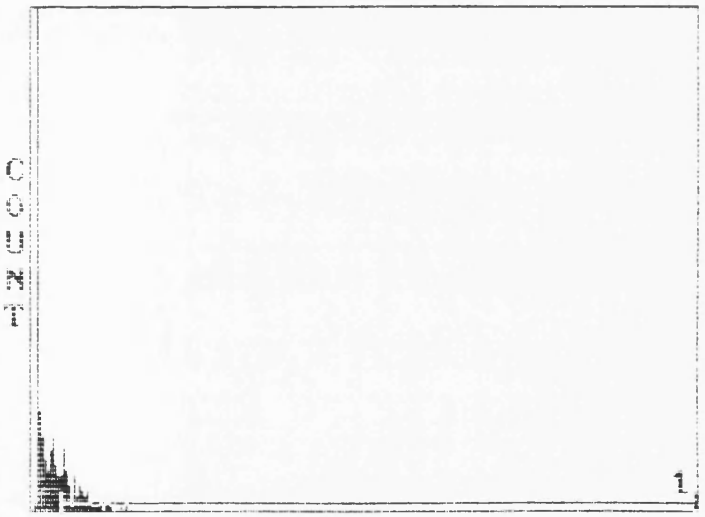




		MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
1	X	1.024	1.415	2034	40.7	1.024	1.031	
	Y	1.577	1023.			2.994	1.348	26.5
2	X	1.416	1023.	1	0.0			
	Y	1.577	1023.					
3	X	1.024	1.415	2904	58.1	1.029	1.054	
	Y	1.024	1.576			1.031	1.068	
4	X	1.416	1023.	60	1.2	1.652	1.201	
	Y	1.024	1.576			1.024	1.024	



	MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
1	1.416	1023.	1644	41.7	3.151	1.369	17.6



	MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
1	1.378	1023.	68	1.7	1.628	1.210	48.2

#245
5:07 p.m.
9-Jun-89
HUMAN LYMPHS
OKT8 -VE CELL

EPICS is a registered trademark of Coulter Corporation.

the contaminating granulocytes, monocytes and B-lymphocytes should not affect the objective of the proliferation assay. ie. that of measuring suppressor cell activity.

2.3.4. Insulin Specific Lymphocyte Proliferation Assay

1 x 10^5 Peripheral blood mononuclear cells suspended in RPMI 1640 medium supplemented with 10 % AB serum and additives, were cultured in 200 ul volume, either in the absence of insulin or in the presence of 10 and 100 ug ml^{-1} human, pork and beef insulins. The assay was performed in triplicate cultures in a 96-well round-bottomed microtiter plate. After 7 days of incubation at 37°C, 5 % CO_2 humid atmosphere, the cells were pulsed with 0.25 uCi $well^{-1}$ ^{3}H-Thymidine (^{3}H-TdR) (specific activity of > 25 Ci $mMol^{-1}$) and incubated for a further 24 hours before harvesting (section 3.3.5).

Non-specific lymphocyte transformation was measured using the mitogen, PHA at a concentration of 1 ug ml^{-1}. The cells were culture in RPMI 1640 medium supplemented with 10 % AB serum and additives. ^{3}H-TdR was added on day two and the cells harvested on day three.

2.3.5. Cell Harvesting and Scintillation Counting

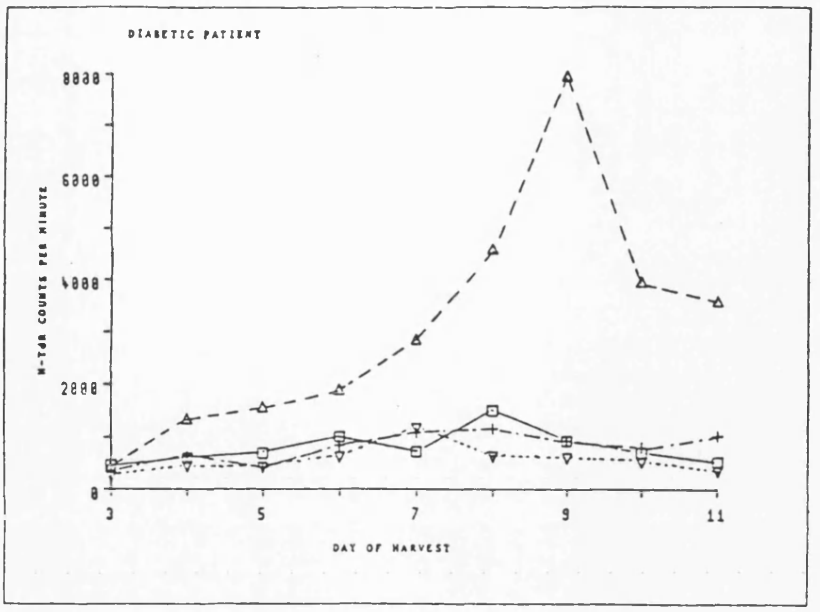
Cells were washed from the 96-well microtiter plate, 12 wells at a time, and deposited on to the filter discs (Titer-tek filter paper) of a memi-automatic Ilacon harvester. The cells were washed with water for 10 seconds followed by 5 % Trichloacetic acid (TCA) for 30 seconds and methanol for 20 seconds. The filter discs were dried overnight at room temperature and placed in plastic insert tubes.

The filter discs were laied flat at the bottom of each insert, and 4 mls of scintillation fluid (Optiphase `Safe`, LKB) was added. The incorporation of ^{3}H-TdR into DNA was determined using a LKB Wallac 1215 Rackbeta Liquid Scintillation Counter.

Optimum culture conditions and kinetics of lymphocyte proliferation responses to insulin.

In order to optimize the *in vitro* lymphocyte responses to insulin, multiple culture parameters were examined by using PBMC from two insulin dependent diabetic patients and a non-diabetic control subject. These parameters included serum source, antigen concentration and kinetics. The resulting protocol which outlines the optimum culture conditions is described in section 2.3.4.

Figure 2.3.

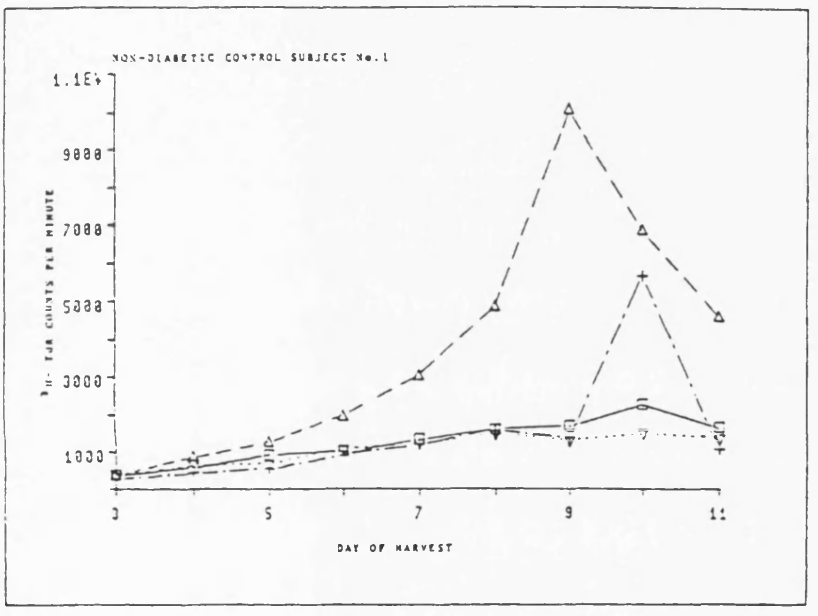


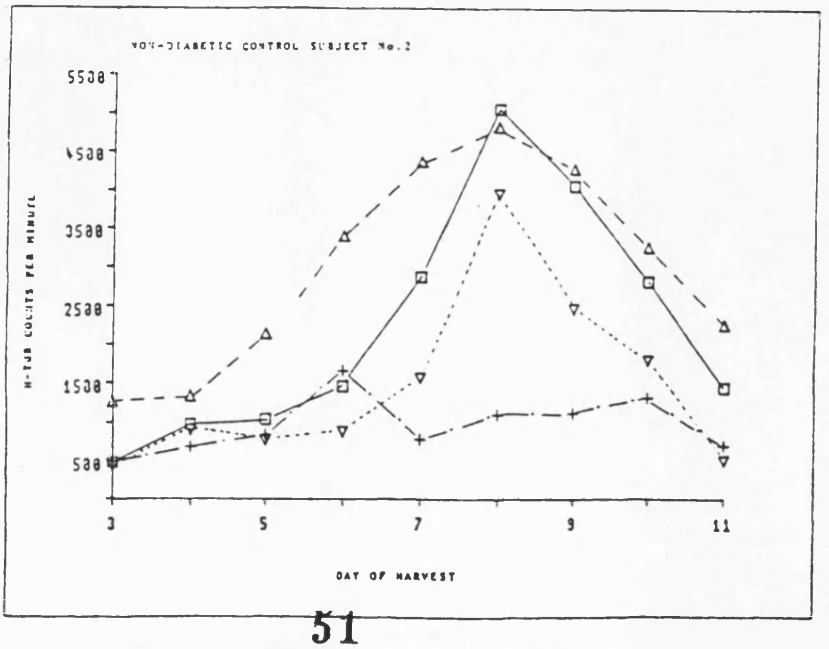
□ NO INSULIN
OKT8 -VE CELLS

△ BOVINE INSULIN
($50\mu g\ ml^{-1}$)
OKT8 -VE CELLS

▽ NO INSULIN
UNFRACTIONATED
CELLS.

+ BOVINE INSULIN
($50\mu g\ ml^{-1}$)
UNFRACTIONATED
CELLS.





Kinetics Of PBMC Proliferation In Response To Insulin

OKT8-ve and unfractionated peripheral blood mononuclear cells (1 x 10^5 $well^{-1}$) from one insulin-dependent diabetic subject (*A) and from two non-diabetic control subjects (*B + *C) were cultured without insulin and with 50 ug ml^{-1} human insulin on day 0. 0.25 uCi $well^{-1}$ ^{3}H-TdR was added for the last 24 hours of culture. The effects of mitogen, Phytohemagglutinin-P (PHA), was also investigated by culturing the cells in 1 ug ml^{-1} PHA.

The results are expressed as mean (± Standard Error (SE)) ^{3}H-TdR uptake of triplicate cultures, and illustrated in Figure 2.3.

Figure 2.3. indicates that optimum cellular proliferation occurs between days 8 and 9. For this reason and because of convenience cells were cultured for 8 days.

Dose Response Of Peripheral Blood Mononuclear Cells To Human, Pork And Beef Insulins

Figures 2.4.(a), (b) and (c) illustrates the PBMC dose response curves to human, pork and beef insulins respectively. Peripheral blood mononuclear cells (PBMC) were cultured with varying concentrations of human, pork and beef insulins for 8 days at 37°C in 5 % CO_2, humid atmosphere. The cells were pulsed with ^{3}H-TdR for 24 hours prior to harvesting. ^{3}H-TdR uptake was determined

by the standard liquid scintillation method. The results are expressed as C.P.M. ± Standard Error (SE).

Figure 2.4. shows a peak response to human and beef insulins at 10 ug ml^{-1} a similar peak is obtained between 80 to 160 ug ml^{-1} with all three types of insulin. Thus the cells were cultured at 10 and 100ug ml^{-1} insulin (see section 2.3.4.).

Foetal Calf Serum (FCS) versus Human AB Serum

OKT8-ve and Unfractionated PBM cells were cultured in RPMI 1640 medium supplemented with Foetal Calf Serum (FCS) or Human AB Serum. All other conditions of the assay were as described in the standard protocol (see section 2.3.4.). The effects of FCS and human AB serum is summerised in Table 2.4.

Table 2.4. shows that a high background reading is obtained with FCS. This has been reported by other workers (Nell et. al., 1983). Human AB serum, in comparison, gives low background readings, and seems to support response to insulin more efficiently. The use of FCS in culture medium was terminated and human AB serum, which was tested for anti-insulin antibodies and found to be negative (< 7.25 ug ml^{-1}), was used instead.

Table 2.4.

Effects of FCS and human AB serum on Stimulation Index (SI) values:

No.	Insulin Conc.	OKT8-ve Cells C.P.M	SI	Unfractionated Cells C.P.M.	SI
*A	0	1957+213	1	1507+215	1
10%	P10	1851+458	0.95	545+72	0.36
FCS	P100	767+181	0.39	909+101	0.60
	B10	1195+303	0.61	290+71	0.19
	B100	551+202	0.28	360+1	0.24
10%	0	862+141	1	522+59	1
AB	P10	2055+225	2.38	426+56	0.82
Serum	P100	1121+239	1.30	1139+380	2.18
	B10	1892+280	2.19	705+365	1.35
	B100	1472+302	1.71	820+32	1.57
*B	0	2112+618	1	1497+260	1
10%	P10	2213+434	1.05	1161+89	0.78
FCS	P100	763+265	0.36	479+129	0.32
	B10	1870+249	0.89	1123+230	0.75
	B100	1195+196	0.57	980+76	0.65
10%	0	579+81	1	1096+251	1
AB	P10	1505+221	2.60	1331+154	1.21
Serum	P100	1234+324	2.13	1240+117	1.13
	B10	1600+123	2.76	1893+154	1.73
	B100	821+150	1.42	1152+96	1.05

**A and *B are diabetic patients. Results are expressed in Counts Per Minute (C.P.M.) ± Standard Deviation. SI=Stimulation Index. Insulin concentration=10 and 100 ug ml^{-1} P=pork and B=beef insulins.*

Figure 2.4.

Peripheral Blood Mononuclear (PBM) Cell Dose Response to Insulin

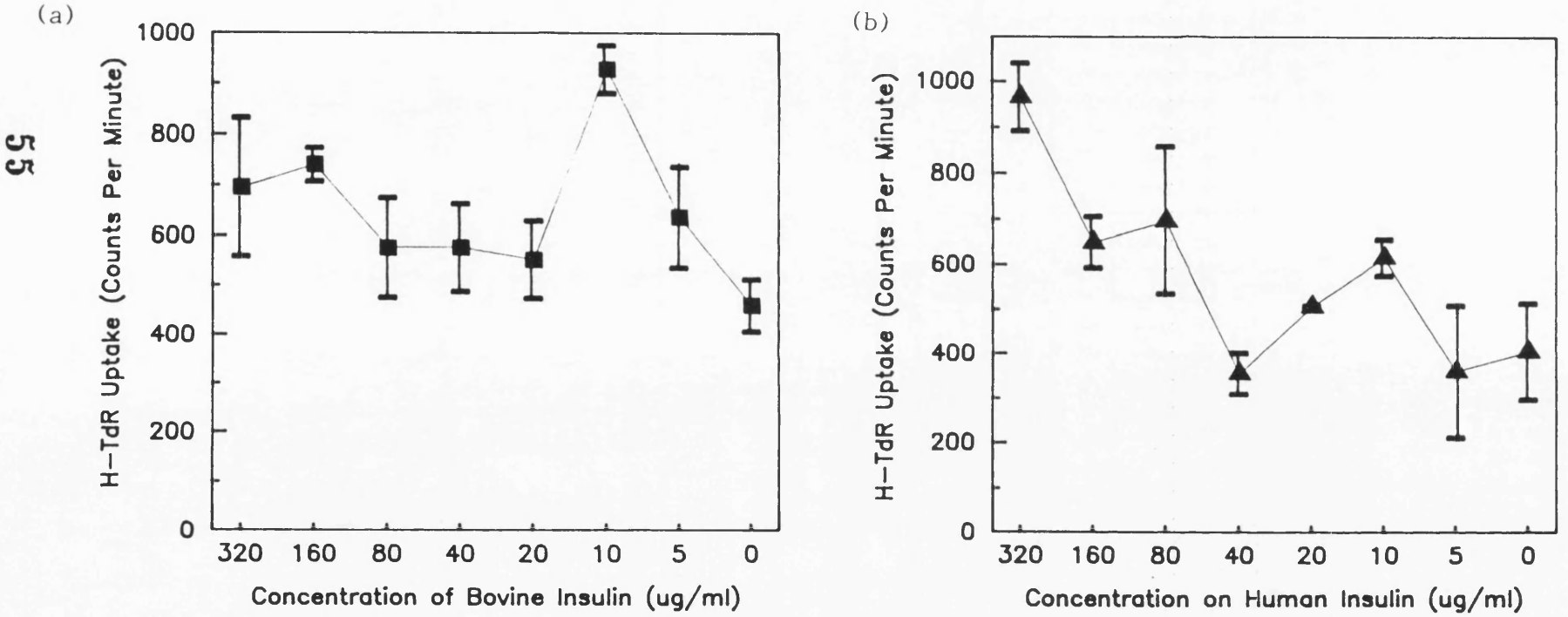
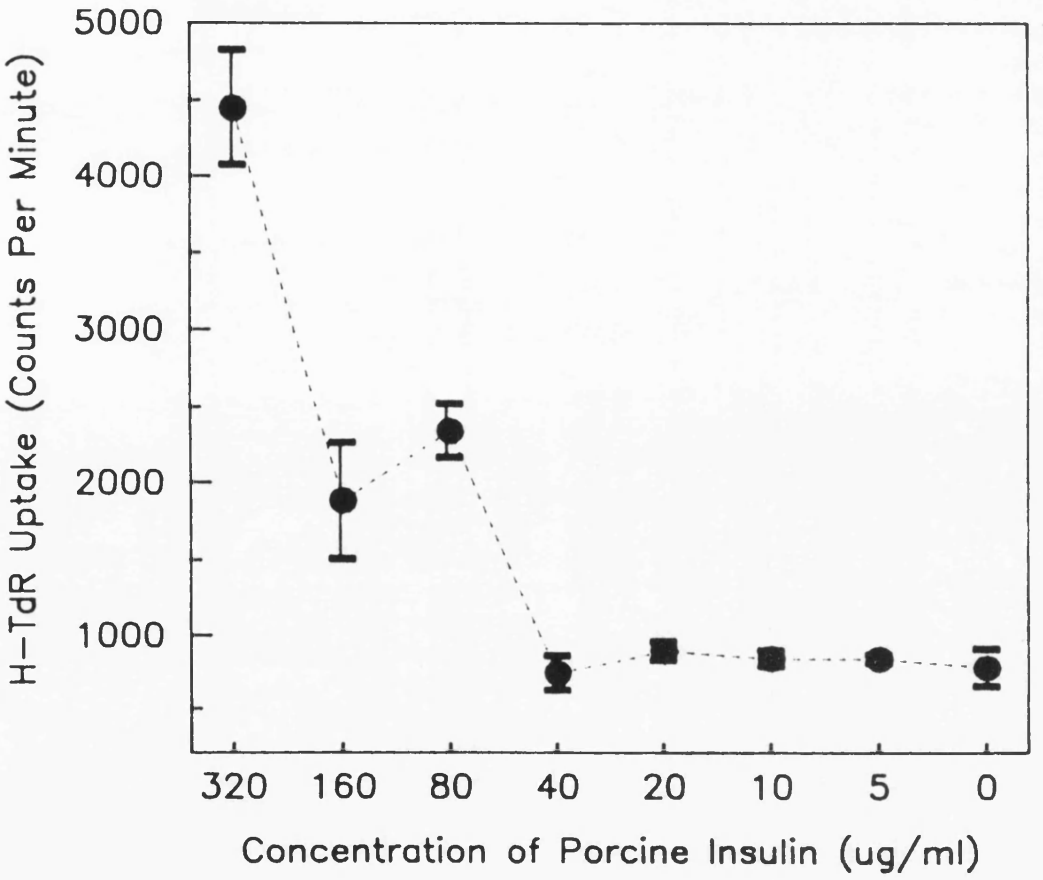


Figure 2.4.(c)

Dose Response of Peripheral Blood Mononuclear Cells to Porcine Insulin



"Edge Effect" of Microtiter Plates.

Depending upon the type and make of a microtiter plate, there is often a variation in readings obtained in the outer wells compared to those of the central wells. This is commonly referred to as the "edge effect" of microtiter plates. It is therefore, necessary to eliminate such variation. The edge effect of Corning polystyrene microtiter plate (Corning Glassworks, Corning, NY) was thus investigated :

5×10^5 cells, in 200ul of RPMI 1640 medium supplemented with 10 % AB serum and additives, were placed into each well of a 96-well round-bottomed microtiter plate.

Following 7 days incubation at 37°C, 5 % CO_2 in humid atmosphere, the cells were pulsed with ^{3}H-TdR for a further 24 hour period.

The cells were harvested on the the 8^{th} day using a semi-automated cell harvester and ^{3}H-TdR incorporation counted using the standard scintillation procedure (section 2.3.5.). This experiment was carried out with 4 different plates. Table 2.5.(a). shows the result of one such plate. All plates gave similar results.

Table 2.5.(a)

"Edge effect" of microtiter plate:

		No.of wells	Mean CPM	Standard Error (SE)	% Coeff. of variation
Consecutive circle of wells, starting from outer circle (A) to the central circle (D) of the plate.	A	36	29590	1179	4.0 %
	B	28	26760	980	3.7 %
	C	20	28577	1238	4.3 %
	D	12	25647	895	3.5 %
Mean CPM of all 4 circles + Standard Deviation (SD)					*= 27644 + 1773.*

Analysis of Variance was carried out to determine whether a significant "edge effect" existed. This is shown in Table 2.5.(b).

Null hypothesis : "The population means xA, xB, xC and xD are different from each other and therefore it follows that there is an "edge effect" of signals produced when Corning microtiter plates are used".

Table 2.5.(b).

Analysis of Variance in readings obtained with consecutive circles of wells in a microtiter plate:

Source of Variation	Degrees of freedom (df)	Sums of Squares (SS)	Variance (or mean square)	Variance Ratio (F)
Total	95	3.37×10^{9}	-	
Between Groups	3	2.07×10^{8}	6.88×10^{7}	2.002
Within Groups	92	3.16×10^{9}	3.44×10^{7}	

Since, $F_{calculated\ value}(2.002) < F_{table}(2.72)$ at P=0.05, the null hypothesis was rejected, i.e. the difference between the means of the four groups, A,B,C and D was not significant (P=0.05). Therefore, the Corning polystyrene microtiter plate did not display any "edge effect" and any variation in signal obtained in future experiments could not be attributed to the physical make-up of the microtiter plate.

Inter- and Intra- Assay analysis

The reproducibility of the insulin specific proliferation assay was determined by taking blood samples from two diabetics (*A & *B) and one control subject (*C) on 4 consecutive months. Unfractionated peripheral blood mononuclear cells were cultured with 10 and 100 ug ml^{-1} human insulin. Results are tabulated in Table 2.6.(a). The coefficient of variation between samples were less than 11% for most cultures indicating that the assay was very reproducible.

The Intra-assay variability was also assessed using the optimal conditions described in section 2.3.4. For each insulin concentration, cells from a diabetic patient (*A), was cultured in 6 replicate wells on the same plate, (see Table 2.6.(b). The coefficeient of variation for intra-assay was less than 15%.

Table 2.6.(a).

Inter-Assay Variability (Stimulation Indices):

Week	Subject *A		*B		*C	
	H10	H100	H10	H100	H10	H100
1	2.21	2.15	2.17	2.49	1.06	1.53
2	1.78	2.39	2.70	2.35	1.25	1.32
3	1.81	2.83	2.63	3.20	1.31	1.49
4	2.07	1.65	2.66	3.08	1.17	1.57
Coeff. of Variation	10.6%	21.8%	9.8%	15.2%	9.0%	7.4%

*Cells cultured in presence of 10 and 100 ug.ml^{-1} H=human insulin. Please note: subjects *A, *B and *C are not the same volunteers referred to in table Table 3.2.*

Table 2.6.(b).

Intra-Assay Variability (Counts Per Minute):

Well No.	OKT8 -ve Cells				Unfractionated Cells			
	0	P10	B10	H10	0	P10	B10	H10
1	2176	4495	3106	3503	2337	4632	3872	3845
2	2303	5250	3407	3788	1722	3666	4420	4084
3	1987	4268	3874	4561	1900	3171	3844	4690
4	1999	4825	4136	4492	2002	4164	2988	4688
5	1845	3911	3033	4388	1926	3659	3306	4335
6	2178	5436	3655	4385	1883	-	3565	3676
*C.V.	8%	12.5%	12.3%	10.4%	10.5%	14.4%	13.6%	10%

*Cells were cultured in the presense of 10ug.ml^{-1} P10=pork insulin; B10=beef insulin; H10=human insulin, or 0=no insulin. *C.V. - Coefficient of Variance.*

2.4. RESULTS

The cellular immune response to insulin was determined using an insulin stimulated lymphocyte transformation assay. It is presumed that the assay measured primary immune response to insulin in normal non-diabetic control subjects and secondary responses in the IDDM patients.

The results are expressed as Stimulation Indices (SI) which were calculated using the following equation:

$$SI = \frac{^{3}H\text{-TdR incorporation (Mean CPM) of triplicate culture with Insulin}}{\text{Mean CPM of triplicate culture without Insulin}}$$

The data were analysed using a general purpose statistical package - `INSTAT` (Ref. Burn R.W., et. al., 1987). The statistical significance in difference of unpaired data were analysed using Students t-test or Mann-Whitney U test depending upon the distribution of the data. Normal distribution of data were assessed by plotting histograms. One of the pre-requisites of a t-test is that the F-ratio must not be significant, ie, the standard deviation of the two groups of data being compared should not be significantly different. Thus where the F-ratio was significant and/or the data did not give a `normal` or `normalizable` distribution, non-parametric tests were used; independent and paired data were analysed using Mann-Whitney U and two-sample Wilcoxon tests respectively.

2.4.1. *Patients on Human Insulin Therapy*

Table 2.7 shows the *in vitro* insulin induced proliferative response (stimulation index (SI)) by non-diabetic control subjects. In table 2.8., the SI values for individual Group I diabetic patients are tabulated. In Table 2.9., SI values of Group I patients and control subjects are compared. The results show that, in the presence of beef (BI) and pork (PI) insulins, there is significant difference in SI of diabetic patients and controls. However, when the cells were cultured in the presence of autologous insulin (human insulin-HI) the difference in response between the two groups was not significant.

When OKT8 +ve cells were removed from the peripheral blood monuclear cells to give a OKT8-ve cell population and cultured in the presence of HI, PI and BI, the SI of diabetic and control subjects did not differ significantly (see Table 2.10).

Table 2.7.

Insulin induced proliferative response (Stimulation Index) in non-diabetic control subjects:

Patient Number	Type & Concentration Of Insulin (in culture) H10	H100	P10	P100	B10	B100
1	0.64	0.96	2.07	1.04	1.40	0.71
2	0.59	0.47	0.40	0.45	0.53	0.35
3	0.48	1.52	1.84	0.70	1.05	0.71
4	0.66	0.47	0.70	0.41	0.64	0.53
5	0.93	2.02	0.62	0.73	1.07	1.86
6	0.70	1.01	0.92	0.82	0.58	0.75
7	0.64	2.19	0.75	1.13	0.90	0.73
8	0.93	1.31	0.79	0.77	0.67	0.72
9	1.28	1.10	1.23	0.99	0.85	0.90
10	0.55	1.18	0.65	0.83	0.73	0.77
11	2.31	1.76	2.37	1.16	2.52	0.95
12	0.61	0.53	0.65	0.79	0.91	0.88
13	1.43	1.20	0.54	3.02	0.84	2.69
14	1.31	0.53	0.48	0.88	1.26	1.06
15	0.72	0.46	0.65	0.86	0.53	0.68
16	0.69	0.88	1.00	0.43	0.45	0.67
17	0.65	0.57	0.41	0.67	0.87	1.12
18	0.84	0.59	0.50	0.45	0.47	0.55
19	1.37	0.79	0.59	0.72	0.89	0.82
20	1.19	0.95	1.56	1.12	0.79	2.10
21	0.48	0.80	0.55	0.97	0.45	0.82
22	2.17	2.29	1.00	0.64	0.90	2.40
N	22	22	22	22	22	22
mean	0.96	1.07	0.92	0.89	0.88	1.04
SD	0.51	0.57	0.56	0.53	0.45	0.63
*PR	>1.98	>2.21	>2.03	>1.94	>1.77	>2.30
median	0.71	0.95	0.68	0.81	0.85	0.80

Cells were cultured in the presence of: H=human insulin; P=pork insulin; B=beef insulin at 10 and 100 $ug.ml^{-1}$. N=number of patients. ND=not determined. SD=standard deviation. *PR=positive response = > mean + 2SD

Table 2.8.

Insulin induced proliferative response (Stimulation Index) in Group I diabetic patients:

Patient Number	Type & Concentration Of Insulin (in culture) H10	H100	P10	P100	B10	B100
1	ND	ND	1.04	0.76	0.88	1.02
2	ND	ND	1.13	1.21	1.05	1.18
3	ND	ND	2.44	2.55	0.62	0.76
4	ND	ND	1.61	1.26	1.49	1.89
5	ND	ND	0.94	0.82	1.09	1.04
6	ND	ND	0.82	2.18	1.35	1.31
7	ND	ND	1.18	1.28	1.34	1.59
8	ND	ND	1.11	0.85	0.95	0.96
9	ND	ND	1.15	2.79	0.69	0.91
10	ND	ND	1.81	2.83	1.65	1.96
11	ND	ND	1.66	2.47	1.79	2.12
12	ND	ND	0.75	0.97	0.49	1.17
13	ND	ND	1.30	1.34	1.13	1.28
14	ND	ND	0.96	0.72	7.26	1.31
15	ND	ND	1.43	0.99	1.27	0.48
16	ND	ND	1.96	1.66	1.45	1.74
17	ND	ND	1.24	1.97	0.67	1.55
18	ND	ND	1.77	1.73	1.12	1.47
19	ND	ND	0.67	1.01	1.33	1.25
20	ND	ND	1.25	2.29	2.51	1.65
21	ND	ND	0.84	0.56	0.51	0.48
22	1.33	1.66	0.65	1.22	1.37	1.49
23	0.74	0.56	0.77	0.80	1.00	0.88
24	1.32	ND	0.48	0.54	0.42	0.47
25	2.00	0.43	1.61	1.27	1.82	2.13
26	0.43	0.47	0.61	0.61	0.43	0.40
27	0.81	0.84	0.49	0.50	0.61	0.55
28	0.54	0.55	0.45	0.46	0.47	0.57
29	2.70	2.02	0.58	0.48	1.41	0.90
30	0.66	0.65	0.76	0.43	0.75	0.76
31	1.08	0.82	1.64	0.84	1.32	1.17
32	1.96	2.38	1.82	1.69	1.39	1.68
33	2.21	2.15	1.93	1.47	2.25	1.96
34	1.58	2.79	2.49	4.24	2.21	4.36
35	4.73	2.45	3.58	3.60	1.65	2.18
36	0.81	0.94	0.77	0.93	0.74	1.12
37	1.49	3.52	2.02	1.65	8.76	3.28
38	2.17	2.22	1.00	0.64	0.90	3.40
39	1.10	1.62	1.94	1.80	1.44	1.73
40	ND	0.93	ND	1.50	ND	1.23
41	ND	0.57	ND	0.50	ND	0.49
42	ND	0.47	ND	0.54	ND	0.66
43	1.68	1.79	0.92	1.05	3.74	1.95
44	0.54	0.63	0.94	1.05	1.01	0.64
45	0.51	0.98	0.90	2.86	1.13	2.77
46	0.91	1.88	0.69	0.69	0.84	2.57

Continued.....

Table 2.8. Continued:

Patient Number	Type & Concentration Of Insulin (in culture) H10	H100	P10	P100	B10	B100
47	1.65	1.10	1.59	1.91	1.30	1.15
48	1.00	0.72	0.96	0.74	1.03	1.25
49	1.04	2.91	1.25	2.75	1.02	2.85
50	0.92	ND	0.66	0.41	0.41	ND
51	2.14	2.35	2.01	1.71	0.95	3.29
52	1.20	1.51	0.90	ND	2.11	ND
53	0.80	1.29	1.09	1.27	2.20	0.40
54	0.84	0.79	1.09	0.73	0.99	0.97
55	0.40	1.02	0.76	1.02	0.47	0.68
56	0.48	0.89	1.18	3.08	0.76	1.93
57	1.07	1.67	1.07	1.76	1.04	1.75
58	0.86	0.85	0.80	3.14	1.11	3.37
59	0.53	ND	0.57	0.88	0.54	0.55
60	1.90	0.94	1.97	1.19	1.63	0.76
61	0.89	0.71	2.00	1.29	0.77	0.99
62	0.78	0.67	0.59	1.35	0.82	1.36
63	1.15	1.84	ND	ND	1.37	1.65
N	39	39	59	61	60	61
mean	1.26	1.35	1.23	1.42	1.41	1.47
SD	0.81	0.80	0.61	0.87	1.38	0.87
median	1.04	0.98	1.09	1.22	1.10	1.25

Cells were cultured in the presence of: H=human insulin; P=pork insulin; B=beef insulin at 10 and 100 $ug.ml^{-1}$. N=number of patients. ND=not determined. SD=standard deviation.

Table 2.9.

Comparison of cellular immune response to insulin by Group I diabetic patients and control subjects: Insulin-specific SI of unfractionated cells.

		Insulin Type & Concentration *in vitro*					
		H10	H100	P10	P100	B10	B100
CONTROLS:	N	22	22	22	22	22	22
	mean	0.96	1.07	0.92	0.89	0.88	1.04
	SD	0.51	0.57	0.56	0.53	0.45	0.63
	median	0.71	0.95	0.68	0.81	0.85	0.80
GROUP I	N	39	39	59	61	60	61
DIABETIC	mean	1.26	1.35	1.23	1.42	1.41	1.46
PATIENTS:	SD	0.81	0.80	0.61	0.87	1.38	0.87
	median	1.04	0.98	1.09	1.22	1.10	1.25
Mann-Whitney	U=	329	355	403	387	409	441
	z=	1.51	1.11	2.62	2.94	2.63	2.37
Probability	>z=	NS	NS	0.009	0.003	0.008	0.018

Cells were cultured in the presence of 10 and 100 ug.ml^{-1} of: H=human insulin; P=pork insulin; B=beef. N=Number of observations. SD=standard deviation. z=standard normal deviate. NS=not significant.

Table 2.10.

Comparison of cellular immune response to insulin by Group I diabetic patients and control subjects: Insulin-specific SI of OKT8 -ve Cell population.

		Insulin Type & Concentration *in vitro*					
		H10	H100	P10	P100	B10	B100
CONTROLS:	N	22	22	22	22	22	22
	mean	1.23	1.18	1.03	1.03	0.98	1.14
	SD	0.64	0.56	0.48	0.61	0.50	0.59
	median	1.07	1.19	0.88	0.91	0.83	0.99
GROUP I	N	39	39	59	61	60	61
DIABETIC	mean	2.42	1.45	1.40	1.75	1.43	1.59
PATIENTS:	SD	6.45	0.88	1.13	1.88	1.38	1.36
	median	1.05	1.19	1.15	1.17	1.10	1.17
Mann-Whitney	U=	385	346	549	495	524	545
	z=	0.67	1.25	1.06	1.82	1.42	1.30
Probability	>z=	NS	NS	NS	NS	NS	NS

Cells were cultured in the presence of 10 and 100 ug.ml^{-1} of: H=human insulin; P=pork insulin; B=beef insulin. N=number of observations. SD=standard deviation. z=standard normal deviate. NS=not significant.

Using an indirect method, insulin specific suppressor cell activity was measured. It is assumed that OKT8 +ve cells carry out most suppressor/cytotoxic functions, thus removal of these cells remove suppressor cell activity. The percentage (%) suppression was measured using the following equation.

$$\% \text{ Supp.} = 100 - \frac{\text{Response (SI) of Unfractionated cells}}{\text{Response (SI) of OKT8 -ve cells}} \times 100$$

The formula makes the following assumptions:

1. A decrease in response by unfractionated cells compared to that of the OKT8 -ve cell population is due to the presence of suppressor (OKT8 +ve) cells and their activity.

2. % suppression may be positive or negative depending upon the ratio of help and suppression which is thought to be carried out by $CD4^+$ and $CD8^+$ ($OKT8^+$) cells respectively.

In Table 2.11., the insulin specific % suppression obtained with cells from Group I diabetic patients are tabulated. In Table 2.12., these results are compared with that obtained with cells from control subjects.

Table 2.12. shows that the insulin-specific suppressor cell activity of diabetic patients and control subjects did not differ significantly.

Table 2.11.

Insulin specific % suppression of Group I diabetic patients:

Patient Number	Type & Concentration Of Insulin (in culture) H10	H100	P10	P100	B10	B100
1	ND	ND	-40.54	-72.73	-120.0	-61.91
2	ND	ND	46.95	53.46	26.06	57.25
3	ND	ND	-106.8	-119.8	19.48	-8.571
4	ND	ND	-30.89	-44.83	-41.91	-85.29
5	ND	ND	-113.6	-64.00	-55.71	-62.50
6	ND	ND	36.92	8.403	21.97	5.755
7	ND	ND	12.59	44.83	-10.74	7.558
8	ND	ND	-68.18	-11.84	-7.955	-41.18
9	ND	ND	28.57	-57.63	71.25	-2.247
10	ND	ND	-43.65	-116.0	-18.71	5.314
11	ND	ND	-43.10	-56.33	2.186	23.74
12	ND	ND	-33.93	11.82	10.91	-7.339
13	ND	ND	-51.16	-127.1	-16.50	-82.86
14	ND	ND	30.94	59.32	11.46	36.10
15	ND	ND	11.73	-8.791	-23.30	50.00
16	ND	ND	6.220	-0.606	-29.46	-83.16
17	ND	ND	-18.10	-38.73	2.899	1.274
18	ND	ND	-58.04	-18.49	4.274	-58.07
19	ND	ND	-81.08	-42.25	-177.1	-140.4
20	ND	ND	-2.459	-10.10	-258.6	26.34
21	ND	ND	0.000	9.677	28.17	46.67
22	37.26	37.12	74.81	59.74	29.38	46.21
23	71.43	52.94	-2.667	23.81	55.95	34.33
24	96.82	ND	93.24	93.84	94.27	68.46
25	37.30	62.28	-292.7	-273.5	-313.6	-232.8
26	48.19	62.10	12.86	8.955	32.81	58.76
27	-52.83	-90.91	-2.083	3.846	7.576	-77.42
28	22.86	24.66	28.57	8.000	17.54	-62.86
29	-315.4	-281.1	-28.89	-23.08	-95.83	-95.65
30	7.042	45.38	-105.4	-26.47	18.49	30.91
31	6.897	18.81	-67.35	40.43	-18.92	30.36
32	7.547	11.85	-41.09	21.03	-21.93	37.08
33	-45.40	10.04	-50.78	-26.72	-82.93	-27.27
34	56.59	-4.494	32.88	-38.11	-13.92	-7.13
35	-729.8	-400.0	-616.0	-471.4	-189.5	-581.3
36	14.74	-36.23	3.750	-34.78	21.28	4.274
37	3.247	-114.6	-29.49	-24.06	-187.2	-54.72
38	13.89	9.016	25.93	46.22	47.98	28.72
39	-23.60	-80.00	-139.5	-119.5	-89.47	-84.04
40	ND	-30.99	ND	-240.9	ND	-68.49
41	ND	34.48	ND	19.36	ND	5.769
42	ND	66.43	ND	61.97	ND	48.03
43	-11.26	-16.23	19.30	-52.17	-605.7	23.83
44	83.13	77.26	74.53	75.97	71.94	80.55
45	73.98	48.96	74.72	66.19	63.78	-264.5
46	-35.82	-108.9	26.60	32.35	-47.37	-182.4

Continued.....

Table 2.11. Continued:

Patient Number	Type & Concentration Of Insulin (in culture) H10	H100	P10	P100	B10	B100
47	*-150.0*	*-80.33*	*-96.30*	*-73.64*	*-154.9*	*-98.28*
48	*-66.67*	*54.14*	*-41.18*	*-15.63*	*-43.06*	*-6.838*
49	*0.952*	*-28.19*	*-8.696*	*-32.21*	*58.20*	*-42.50*
50	*63.78*	*ND*	*76.34*	*39.71*	*66.12*	*ND*
51	*-23.70*	*12.31*	*18.95*	*23.66*	*12.84*	*-51.61*
52	*19.46*	*-75.58*	*13.46*	*ND*	*-70.16*	*ND*
53	*59.39*	*64.46*	*65.72*	*5.93*	*-5.769*	*77.65*
54	*16.83*	*57.53*	*41.40*	*68.26*	*46.77*	*59.41*
55	*40.30*	*-37.84*	*5.000*	*45.16*	*24.19*	*32.67*
56	*33.33*	*4.301*	*51.04*	*23.00*	*49.33*	*38.92*
57	*-8.081*	*45.78*	*-25.88*	*17.37*	*19.38*	*-13.64*
58	*8.511*	*44.81*	*50.00*	*67.86*	*10.48*	*63.57*
59	*17.19*	*ND*	*-42.50*	*-3.529*	*-22.73*	*77.99*
60	*19.49*	*-100.0*	*-42.75*	*56.25*	*-18.98*	*41.54*
61	*-7.229*	*57.23*	*-56.25*	*-10.26*	*39.37*	*6.604*
62	*58.29*	*-17.54*	*-37.21*	*-58.82*	*-26.15*	*-27.10*
63	*-13.86*	*-159.2*	*ND*	*ND*	*-44.21*	*-81.32*
N	39	39	59	61	60	61
mean	-14.5	-19.5	-24.7	-20	-30.4	-25
SD	136.7	97.8	101	90	109.8	102
median	13.9	10	-2.7	-3.5	-1.8	1.3

Cells were cultured in the presence of: H=human insulin; P=pork insulin; B=beef insulin at 10 and 100 $ug.ml^{-1}$. N=number of patients. ND=not determined. SD=standard deviation.

Table 2.12.

Comparison of % Suppression of non-diabetic controls and Group I IDDM patients.

		Insulin Type & Concentration *in vitro*					
		H10	H100	P10	P100	B10	B100
CONTROLS:	N	22	22	22	22	22	22
	mean	9.39	-0.8	-14.6	-7.4	-16	0.69
	SD	43.3	46.6	95.8	67.7	110	49
	median	18	7.6	24	7.4	17.9	9
GROUP I PATIENTS:	N	39	39	59	61	60	61
	mean	-14.5	-19.5	-24.7	-20	-30.4	-25
	SD	136.7	97.8	101	90	109.8	102
	median	13.9	10	-2.7	-3.5	-1.8	1.3
Mann-Whitney	U=	426	429	537	634	550	588
	z=	0.05	0.33	1.19	0.39	1.15	0.86
Probability	>z=	NS	NS	NS	NS	NS	NS

Non-parametric two-sample test for independent data (Mann-Whitney U test). H=human insulin; P=pork insulin; B=beef insulin at 10 and 100 ug.ml^{-1}.

2.4.1.(a) *Insulin Stimulated Cellular Proliferative Response Of Group I Diabetic Patients*

The differential secondary immune response to different types and concentration of insulin in culture were examined. In Table 2.13. the effect of insulin concentration on the SI of unfractionated and OKT8 -ve cells are assessed using a two-sample Wilcoxon test for paired data.

In most diabetic patients the SI is higher with 100 $ug.ml^{-1}$ than with 10 $ug.ml^{-1}$, ie. there is a dose response. However this dose response effect is only significant with beef insulin. Interestingly, the removal of OKT8 +ve cells from culture gives a significant dose response with all three species of insulin.

Proliferative response to human, pork and beef insulins were compared. This is shown in Table 2.14

Table 2.13.

Effects of insulin concentration on SI:

Insulin concentration		Unfractionated Cells H	P	B	OKT8 -ve Cells H	P	B
	N	36	56	58	36	56	58
10 ug.ml^{-1}	mean	1.29	1.27	1.45	1.43	1.3	1.35
	SD	0.86	0.61	1.42	0.86	0.82	1.19
100 ug.ml^{-1}	mean	1.44	1.5	1.53	1.64	1.72	1.66
	SD	0.83	0.88	0.87	0.84	1.7	1.41
Wilcoxon T=		243	666	553	191	521	606
SD=		63.7	122.6	129.2	58.5	129	129
z=		1.41	1.07	2.34	2.28	2.76	2.31
Probability=		NS	NS	0.01	0.023	0.006	0.02

z=standard normal deviate. NS=Not Significant. SI obtained with: H=human insulin; P=pork insulin; B=beef insulin.

Table 2.14.

Difference in lymphocyte proliferative responses (SI) to human, pork and beef insulins. Mann-Whitney U test:

		Type & Concentration of Insulin *in vitro* H10	H100	P10	P100	B10	B100
	N =	39	39	59	61	60	61
H10	U =		708	1151	1142	1009	1178
N=39	z =		0.53	0.82	0.34	1.16	0.08
H100	U =	703		952	1068	935	1141
N=39	z =	0.58		1.44	0.86	1.69*	0.35
P10	U =	1107	1108		1665	1770	1628
N=59	z =	0.31	0.31		0.71	0.27	0.90
P100	U =	1065	1155	1656		1670	1808
N=61	z =	0.88	0.24	0.73		0.83	0.27
B10	U =	1127	1127	1770	1696		1608
N=60	z =	0.31	0.31	0.03	0.70		1.15
B100	U =	1014	1108	1567	1790	1596	
N=61	z =	1.24	0.58	1.22	0.36	1.22	

*Cells cultrured in presence of: 10 and 100 ug.ml^{-1} H=human, P=pork and B=beef insulins. * P<0.05 U=Mann-Whitney U value. z=standard normal deviate. Top right triangle refers to OKT8-ve cells; bottom left triangle refers to unfractionated cells.*

Table 2.15. shows that there is no detectable difference in the overall proliferative response with regards to species of insulin and/or concentration. Sequential comparison of individual patient's data using a two-sample Wilcoxon test for paired data showed that at 100 $ug.ml^{-1}$, the SI response to beef insulin was significantly higher than with human insulin ($P<0.05$). However, correlation coefficient determinations between human, pork and beef insulin-induced SI (and % Suppression) in group I diabetic patients showed a highly significant correlation between the three responses (see Table 2.15.).

Comparison of the SI of OkT8-ve cell populations and the corresponding unfractionated cells showed no significant difference.

Table 2.15.

Spearman's Rank Correlation Coefficient (r_s) of SI and % suppression between the three types of insulin.

			Stimulation Index (SI)					
			H10	H100	P10	P100	B10	B100
% Suppression	H10	N=			34		34	
		r=			0.54**		0.67***	
	H100	N=				34		34
		r=				0.46**		0.62***
	P10	N=	34				55	
		r=	0.52***				0.47**	
	P100	N=		34				55
		r=		0.33^{NS}				0.57***
	B10	N=	34		55			
		r=	0.56***		0.53**			
	B100	N=		34		55		
		r=		0.49**		0.55***		

***=P<0.01; ***=P<0.001; r=Spearman's rank correlation coefficient value. The top right triangle shows the correlation in SI obtained with 10 and 100 ug.ml^{-1} H=human; P=pork; and B=beef insulins. The Bottom left triangle gives the correlation in % suppression obtained with the three types of insulin in culture.*

2.4.1.(b) ***Relationship Between Patient's Clinical Background and Cellular Immune Response to Insulin.***

One of the main objectives of this thesis was to investigate whether the *in vitro* cellular immune response to the various types of insulin would have any clinical relevance, ie. was there a relationship between insulin-specific stimulation index (and % suppression) and the clinical background of the patients.

Patients 'age', 'duration of disease', 'duration of insulin therapy' and 'daily insulin dose' were all considered for their potential effect on the cellular immune response to insulin. No correlation existed between these factors and stimulation index using unfractionated cells.

There was, however, a significant correlation between suppressor cell activity (measured as % suppression) at 10 $ug.ml^{-1}$ human insulin and patients' 'Age'. This is illustrated in Figure 2.5., which shows a decrease in suppressor cell activity with age. Spearman's rank correlation coefficient values for % suppression and clinical background are tabulated in Table 2.16.

A statistically significant correlation between patients' daily insulin dose and % suppression was also observed when the patients cells were cultured with 10 $ug.ml^{-1}$ human insulin. This is illustrated in Figure 2.6.(a). Computer assisted Spearman's Rank Correlation Coefficient test was used to determine the significance level.

Kendel's Rank Correlation Coefficient (Wardlaw A.C., 1985) was determined by hand for verification. Almost identical results were obtained.

This correlation was even stronger, when only patients with extremely high (>1.06 U/D/Kg) and low (<0.407 U/D/Kg)) daily insulin dose requirement were used. Thus when data for patients with mean dose + SD (0.73 (U/D/Kg) + 0.33) were removed prior to analysis, the following correlation emerged: r=0.867; N=11; P<0.001). Figure 2.6.(b) illustrates the relationship between % suppression and patients daily insulin dose requirement.

Table 2.16.

Relationship between % suppression and patients' clinical background. Spearman's Rank Correlation Coefficient (r_s):

	Insulin Type & Concentration *in vitro*					
	H10	H100	P10	P100	B10	B100
N	34	34	55	55	55	55
AGE (years)	-0.36 P<0.05	-0.08 NS	-0.08 NS	0.13 NS	-0.13 NS	-0.17 NS
DURATION OF DISEASE (yrs)	-0.12 NS	-0.18 NS	-0.04 NS	0.03 NS	0.07 NS	-0.05 NS
DURATION OF INSULIN THERAPY	-0.12 NS	0.19 NS	-0.04 NS	0.03 NS	0.07 NS	-0.05 NS
DAILY INSULIN DOSE (U/D/Kg)	0.387 P<0.05	0.292 NS	-0.02 NS	-0.02 NS	0.06 NS	0.03 NS
N	12	12	15	15	15	15
DOSE*	0.867 P<0.001	0.504	-0.04	-0.05	-0.05	0.39

** Values for patients on high (>1.06 U/D/Kg) and low (<0.407 U/D/Kg) insulin dose only, (U/D/Kg=units/day/Kg body weight). H=human insulin; P=pork insulin; B=beef insulin at 10 and 100 ug.ml^{-1}. NS=not significant.*

Figure 2.5.

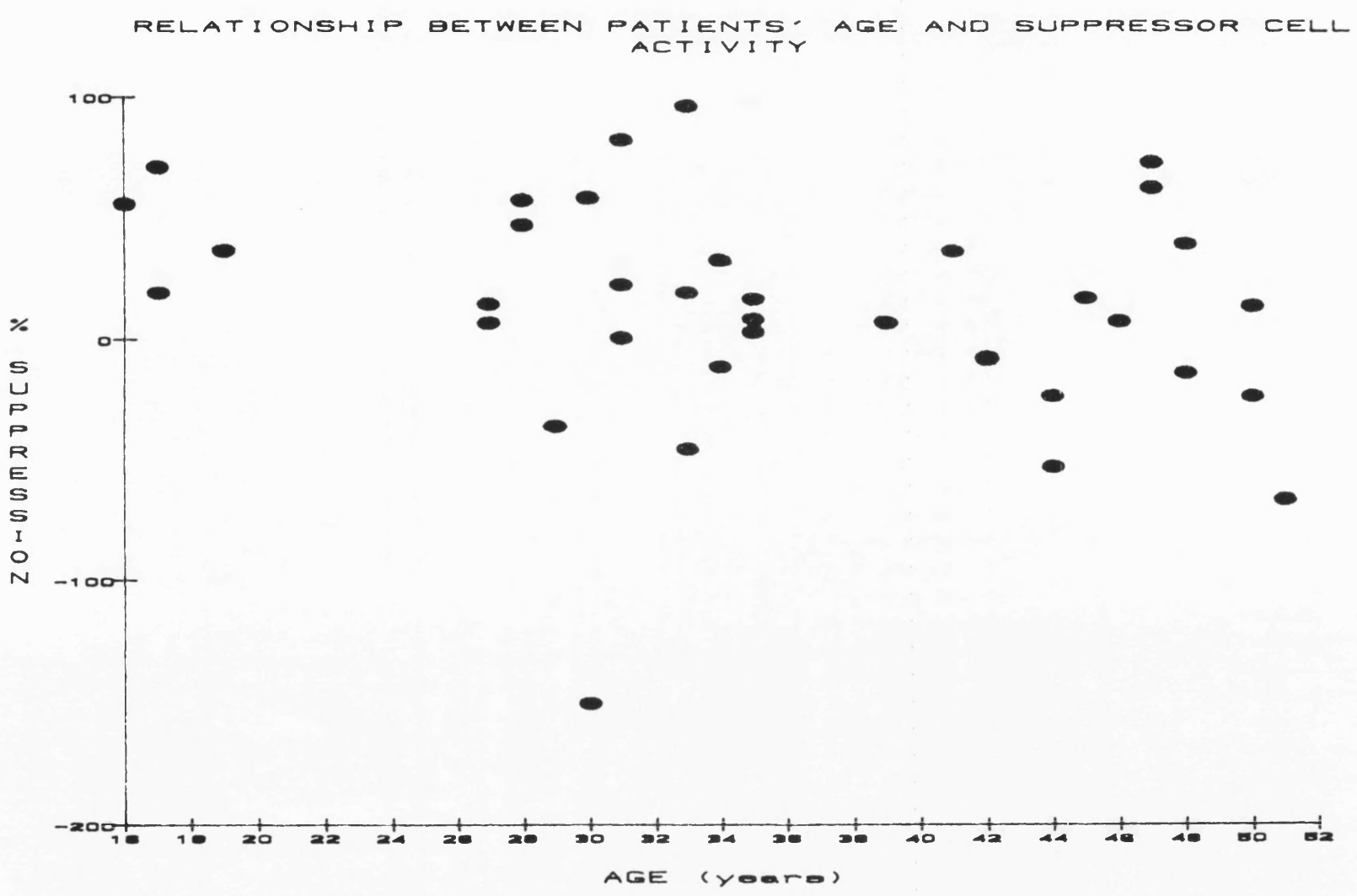

RELATIONSHIP BETWEEN PATIENTS' AGE AND SUPPRESSOR CELL ACTIVITY
% SUPPRESSION
100
0
-100
-200
16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50 52
AGE (years)
% SUPPRESSION WITH 10ug/ml HUMAN INSULIN
N=37
r=-0.36
P<0.05

Figure 2.6(a)

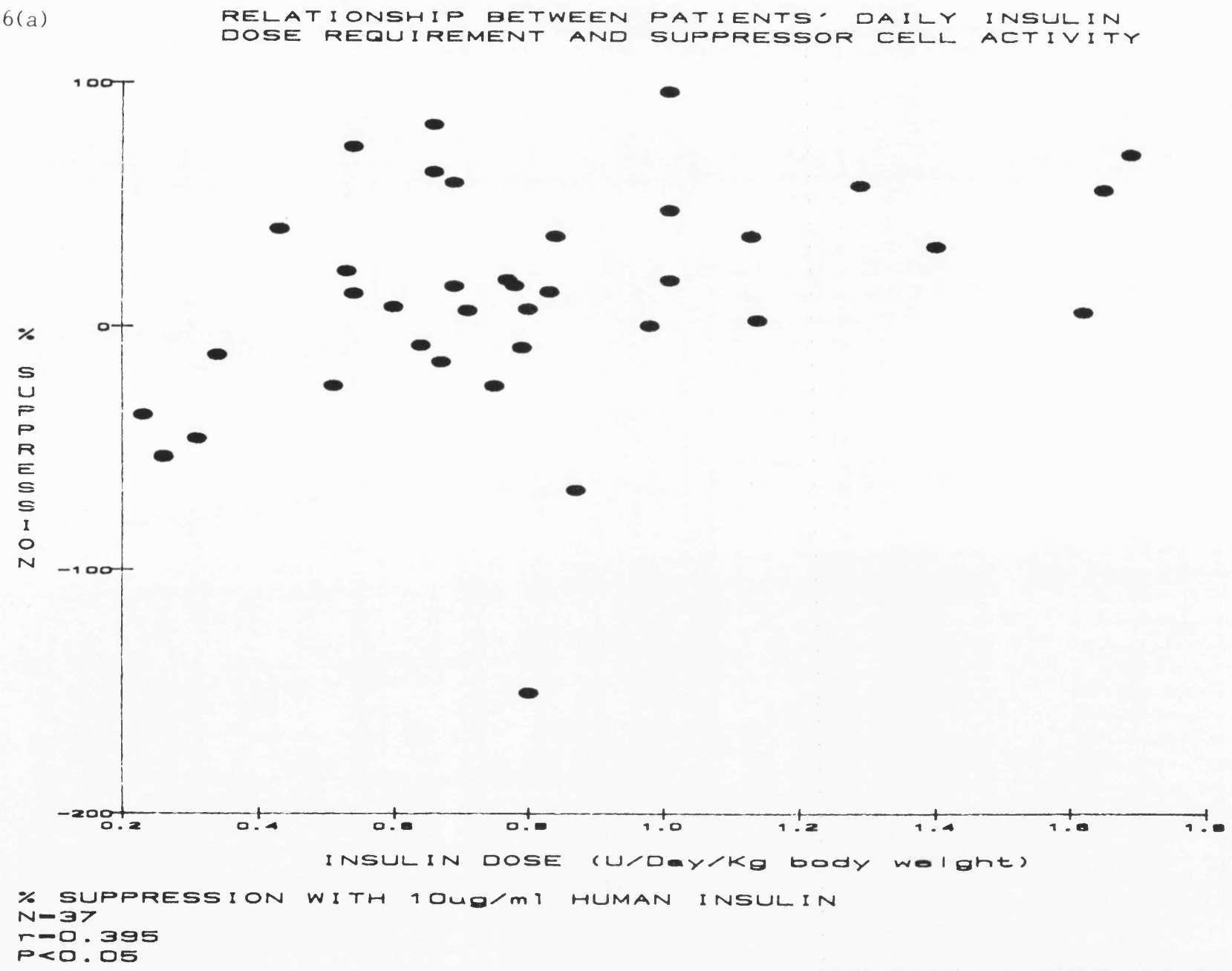
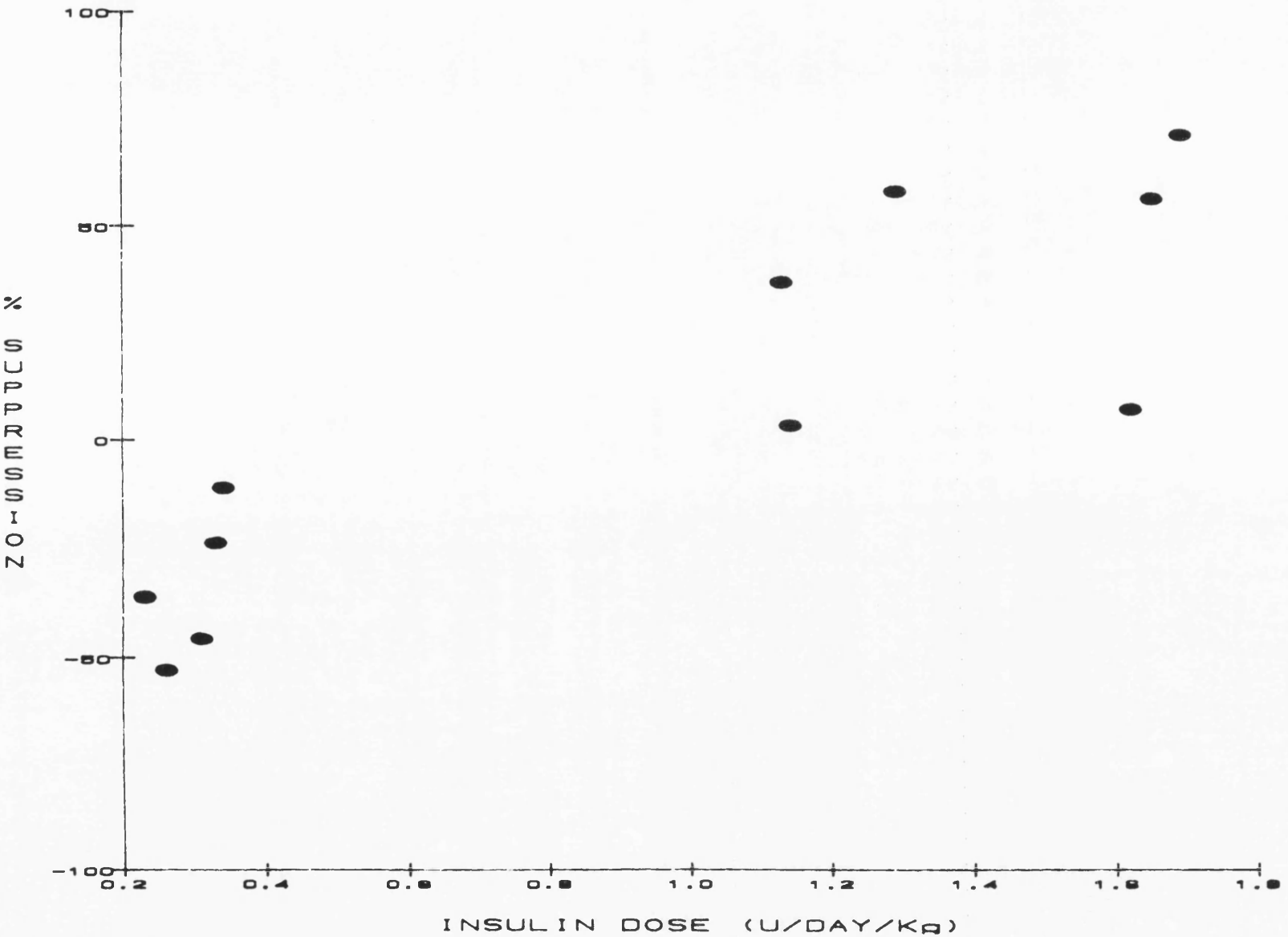

RELATIONSHIP BETWEEN PATIENTS' DAILY INSULIN DOSE REQUIREMENT AND SUPPRESSOR CELL ACTIVITY
100
0
-100
-200
% SUPPRESSION
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
INSULIN DOSE (U/Day/Kg body weight)
% SUPPRESSION WITH 10ug/ml HUMAN INSULIN
N=37
r=0.395
P<0.05

Figure 2.6(b).

RELATIONSHIP BETWEEN PATIENTS' DAILY INSULIN DOSE REQUIREMENT AND SUPPRESSOR CELL ACTIVITY (PATIENTS ON HIGH AND LOW INSULIN DOSE ONLY).



Finally, in order to investigate the effects of cellular immune response (SI) and suppressor cell activity (% suppression) on the patients' diabetic control (measured in terms of percentage glycosylated heamoglobin - % HbA1), Group I diabetic patients were divided into two sub-groups on the basis of their % HbA1 value. Table 2.17. shows the results of a two-tailed non-parametric test for independent data (Mann-Whitney U test).

Blood samples were taken for HbA1 determination on the same day proliferation assays were performed. HbA1 was measured by the column chromatographic technique of Kynoch and Lehmann (1977) by clinical research technicians, department of immunology, Royal United Hospital (R.U.H).

The mean % HbA1 value for Group I patients was 11.7 %. Patients with % HbA1 value of < 11 % are said to have good diabetic control and those with a % HbA1 value >13 % are said to show poor diabetic control (Dr. Reckless, Consultant Physician, R.U.H., Bath).

Table 2.17. shows that the SI, in the presence of all three types of insulins (at 100ug.ml^{-1}), was significantly higher in patients with poor diabetic control than in patients with good diabetic control. The % suppression was found not to be affected by diabetic control, or vice versa.

Table 2.17.

Comparison of SI of patients with good and poor diabetic control:

		Insulin Type & Concentration *in vitro*					
		H10	H100	P10	P100	B10	B100
PATIENTS WITH	N	11	12	17	19	17	18
GOOD DIABETIC	mean	1.18	1.24	1.04	1.12	1.09	1.24
CONTROL	SD	0.75	0.67	0.44	0.66	0.46	0.55
<11% HbA1	median	0.92	0.93	0.94	0.99	1.09	1.21
PATIENTS WITH	N	8	8	16	16	16	16
POOR DIABETIC	mean	1.61	2.02	1.42	1.91	2.14	1.84
CONTROL	SD	1.38	0.98	0.75	1.13	2.39	1.15
>13% HbA1	median	1.27	2.25	1.13	1.81	1.39	1.76
Mann-Whitney U =		37.5	21	87	89	102	83.5
z =		0.54	2.08	1.77	2.09	1.22	2.09
Probability (P)=		NS	0.04	0.078*	0.037	NS	0.037

*H=human insulin; P=pork insulin; B=beef insulin at 10 and 100 ug.ml^{-1}. *=not significant.*

2.4.1.(c) **Results Of Individual Patients**

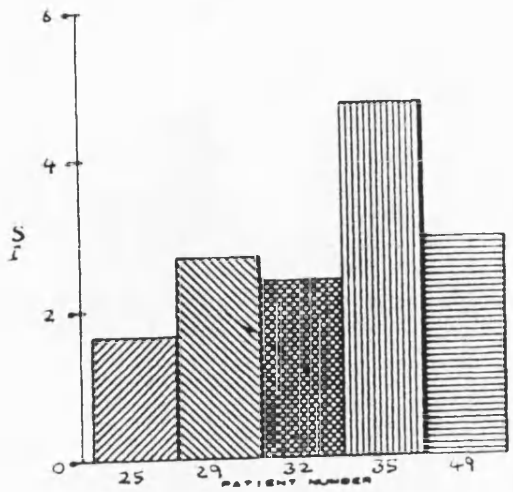
A Positive Response was defined as one which exceeded the control (SI) response to insulin by more than 2 standard deviations. In Table 2.7. the `positive response' values of each ligand and concentration are also tabulated.

In Table 2.8. the SI of individual Group I patients are listed. It show that approximately 40% (25 out of 63) diabetic patients on human insulin therapy gave positive proliferative response (SI) to at least one type of insulin. In comparison, approximately 18% (4 out of 22) control subjects gave a positive response.

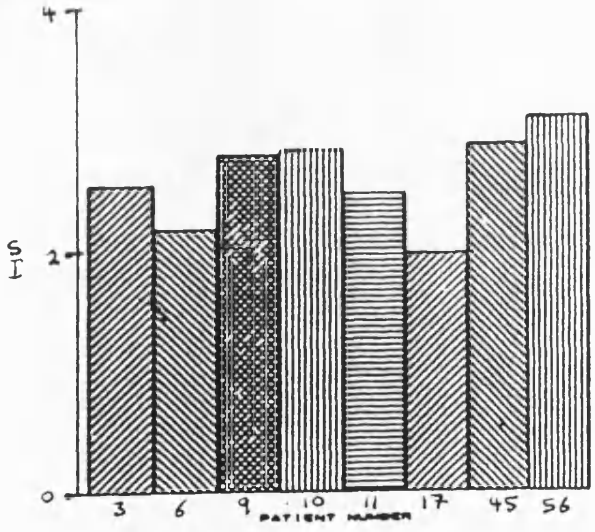
Of the positive responders, 24% (10 out of 41) gave positive response to human insulin, 21% (13 out of 63) responded to pork insulin and 24% (15 out of 63) responded to beef insulin. In Figure 2.7., stimulation indices of patients giving maximum responses to human, pork and beef insulins are illustrated. It shows that, although the proportion of patients giving a positive response to all three types of insulin is similar, the majority (12 out of 25) of patients show a maximum response to beef insulin.

Figure 2.7.

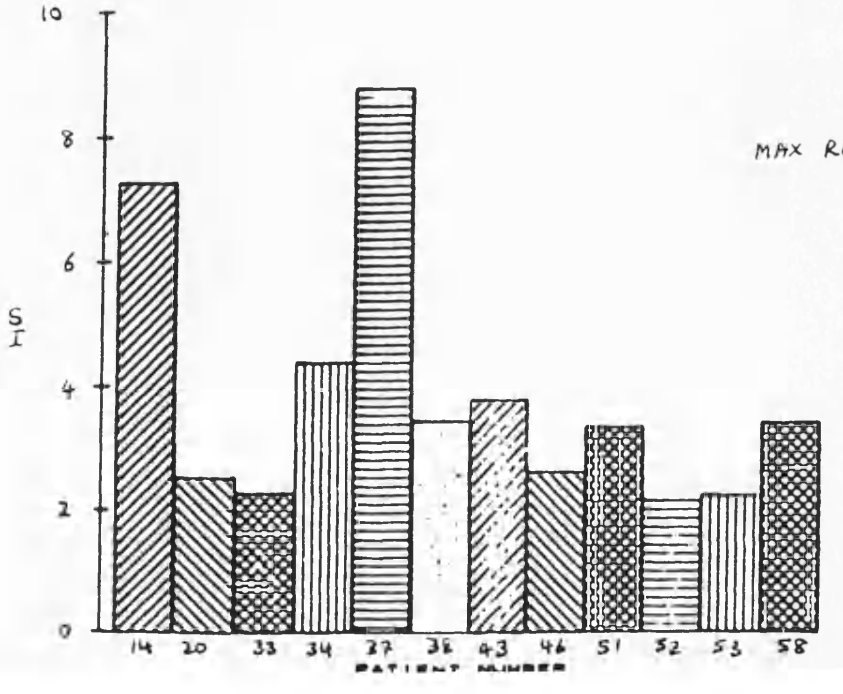
CELLULAR IMMUNE RESPONSE TO INSULIN
MAXIMUM STIMULATION INDEX (SI) TO HUMAN, PORK & BEEF INSULINS



MAX RESPONSE TO HI



MAX RESPONSE TO PI



MAX RESPONSE TO BI

2.4.1.(d) Responder Status

A positive responder (as opposed to a `positive response`) was defined as any individual who gave a positive response to at least one type of insulin. On the basis of this definition, group I diabetic patients were divided into two sub-groups: Responders and Non-responders.

Patients' `age', `diabetic control', `dose', `duration of insulin therapy' and `duration of disease' were all considered to have a potential relationship with the *in vitro* insulin specific-suppressor cell activity and insulin stimulated lymphocyte proliferation. These factors were considered in relationship to the level of stimulation and % suppression (discussed earlier see Table 2.16.) and to the category of response and non-response, see Tables 2.18. and 2.19.

Although the difference in `age', `duration of disease', `duration of insulin therapy' and `dose' was not significant between the two groups, responders were found to have significantly higher HbA1 values than non-responders ($P<0.001$). This reiterates results discussed previously (ie. Table 2.17. shows that responsiveness is associated with poor diabetic control).

Group I patients were also divided into two further sub-groups: those giving positive suppression and those giving a negative % suppression value with human insulin (10 and 100ug.ml^{-}). In Table 2.19. the clinical

background of patients with positive and negative % suppression are compared.

Patients showing human insulin-specific suppressor cell activity were found to have a higher daily insulin dose requirement than those who showed no suppression (i.e. negative suppression).

Table 2.18.

Relationship between patients' clinical background and responder status:

		Patients' Clinical Background					
		Age	DID	DIT	-->HI	Dose	HbA1
RESPONDERS:	N	24	24	24	24	24	20
14 male;	mean	37.3	14.4	13.6	1.42	0.69	12.8
10 female.	SD	10.7	11.2	11	0.81	0.29	2.35
	median	35	10.5	10.5	1.17	0.64	13.4
NON-RESPONDERS:	N	37	37	37	37	37	30
19 male;	mean	35.5	13.2	12.4	1.76	0.80	10.99
18 female.	SD	9.79	8.93	8.53	1.55	0.38	2.05
	median	35	12	11	1.0	1.77	11.05
Mann-Whitney	U	388	432	423	444	342	144
	z	0.83	0.18	0.32	0.01	1.51	3.1
	P	NS	NS	NS	NS	NS	0.002

Non-parametric test (Mann-Whitney U) testing difference in median of the two group=0. DID=Duration of Disease(years); DIT=Duration of Insulin Therapy(years); -->HI=Duration of Human Insulin therapy (years); HbA1=% glycosylated haemoglobin; Dose (units/day/Kg) N=Number of patients. P=probability, significance level. z=standard normal deviate.

Table 2.19.

Relationship between suppressor cell activity (% suppression) and patients' clinical background.

		Patients' Clinical Background				
		Age	DID	DIT	Dose	% HbA1
PATIENTS WITH +VE SUPPRESSION:	N	16	16	16	16	18
	mean	32	15.4	15.4	0.90	12.07
	SD	11	10	10	0.41	2.25
	median	32.5	13	13	0.70	12.2
PATIENTS WITH -VE SUPPRESSION:	N	8	8	8	8	6
	mean	38.5	12.9	11.8	0.42	10.63
	SD	8.59	11.1	11.1	0.2	2.41
	median	37	11	11	0.34	11.4
Mann-Whitney	U	45.5	54	52	13.5	35
std. normal deviate:	z	1.13	0.58	0.74	3.09	1.27
significance level:	P	NS	NS	NS	0.002	NS

N=number of patients. DID=duration of disease (years); DIT=duration of insulin therapy (years); Dose (U/Day/Kg body weight); % HbA1=glycosylated haemoglobin. SD=standard deviation.

2.4.2. *Patients Transferred From Bovine Insulin To Human Insulin Therapy*

Group II individuals consisted of IDDM patients who were transferred from beef insulin to human insulin therapy. Blood samples were taken while the patients were being administered beef insulin, 3-8 months after they were transferred to human insulin, and 12 months later.

The effects of this change in therapy on insulin stimulated lymphocyte proliferation and suppressor cell activity were investigated.

2.4.2.(a) *Cellular Immune Response (SI) Of Group II Patients And Non-diabetic Controls.*

The statistics of SI values of Group II diabetic patients are tabulated in Table 2.20. The difference in SI of Group II patients are compared with those of controls.

The relatively low SI values obtained with Group II patients' 1st samples, are probably due to the use of 10% Foetal Calf Serum (FCS) in the culture medium. It has been shown that FCS supports insulin-specific lymphocyte proliferation very poorly, (see 'Methods' section, Table 2.4. "Foetal Calf Serum (FCS) versus human AB Serum"). FCS also gives high background readings in the absence of

insulin, which makes it impossible to determine how much of the stimulation is due to FCS and how much due to insulin. Unfortunately, human AB serum, which was found to be more appropriate, was not available during analysis of the first samples. The second and third samples were analysed using 10% human AB serum in the culture media. Table 2.20. shows that the SI values obtained with the diabetic patients' first samples did not differ significantly from that of the control subjects. The second and third samples did however give significantly higher SI values than control subjects (with all three types of insulin).

In Table 2.21, the difference in % suppression of Group II patients and controls are analysed. It shows that, in the presence of pork and beef insulins, the % suppression of Group II diabetic subjects did not significantly differ from that of control subjects. In the presence of 10 $ug.ml^{-1}$ human insulin, however, the % suppression values of 2nd ($P=0.05$) and 3rd samples ($P=0.02$) did significantly differ from control subjects. Unfortunately, the suppressor cell activity of the 1st samples were not determined because human insulin was not available at the time of blood sampling.

Table 2.20.

Statistical summary of insulin-specific SI of unfractionated cells (Group II patients); Comparison with SI of controls.

		Type & Concentration of Insulin *in vitro*					
		H10	H100	P10	P100	B10	B100
CONTROLS:	N	22	22	22	22	22	22
	mean	0.96	1.07	0.92	0.89	0.88	1.04
	SD	0.51	0.57	0.56	0.53	0.45	0.62
	median	0.71	0.95	0.68	0.81	0.85	0.80
GROUP II	N	-	-	22	22	22	22
PATIENTS:	mean	-	-	0.97	1.19	0.92	1.03
1st	SD	-	-	0.44	1.19	0.57	0.69
SAMPLE	median	-	-	0.92	1.00	0.80	0.86
Mann-Whitney	U	-	-	192	169	233	223
	z	-	-	1.17	1.73	0.22	0.46
	P	-	-	NS	NS	NS	NS
2nd SAMPLE	N	22	22	22	22	22	22
	mean	1.58	1.33	1.29	1.27	1.49	1.65
	SD	1.5	0.77	1.25	0.7	1.92	2.1
	median	1.07	1.20	0.97	1.14	1.05	1.13
Mann-Whitney	U	138	170	155	158	139	181
	z	1.87	1.02	2.05	1.98	2.43	1.44
	P	0.06*	NS	0.04	0.047	0.015	NS
3rd SAMPLE	N	22	22	22	22	22	22
	mean	1.92	2.05	1.1	1.49	1.49	1.76
	SD	2.31	0.65	0.48	0.63	1.19	2.21
	median	1.07	0.98	0.97	1.61	1.12	1.03
Mann-Whitney	U	147	210	162	86	125	183
	z	1.85	0.25	1.47	3.37	2.39	0.93
	P	NS*	NS	NS	0.0008	0.017	NS

*The Mann-Whitney U test. * P = 0.06 (N.B. human insulin was not available during the analysis of 1st samples). 1st Samples: prior to change from BI to HI therapy; 2nd Samples: 3-8 months after transferring to HI therapy. 3rd Samples: >12 months on HI therapy. H=human insulin; P=pork insulin; B=beef insulin at 10 and 100 ug.ml^{-1}. SD=standard deviation, z=standard normal deviate, P=probability.*

Table 2.21.

Comparison of the % suppression of Group II patients and control subjects:

		Insulin Type & Concentration *in vitro*					
		H10	H100	P10	P100	B10	B100
CONTROLS:	N	22	22	22	22	22	22
	mean	9.39	-0.84	-14.6	-7.38	-15.98	3.58
	SD	43.3	46.6	95.8	67.6	110	37.4
	median	18	7.62	24.2	7.44	17.89	9.06
1st SAMPLE.	N	-	-	22	22	22	22
	mean	-	-	-1.91	-19.6	2.70	-10.7
	SD	-	-	53.5	53	47.1	66.4
	median	-	-	12.6	-7.28	16.98	8.71
Mann-Whitney	U	-	-	224	2.09	234	228
	z	-	-	0.42	0.78	0.19	0.34
	P	-	-	NS	NS	NS	NS
2nd SAMPLE.	N	19	19	22	22	22	22
	mean	-42	-2.65	-32.5	-12.1	-9.19	-31.2
	SD	93.4	65.4	87.4	66.4	55.6	94.3
	median	-8.25	10.84	-24.1	1.75	13.7	-2.74
Mann-Whitney	U	135	1.88	200	237	242	217
	z	1.93	0.55	0.99	0.12	0.47	0.6
	P	0.053	NS	NS	NS	NS	NS
3rd SAMPLE.	N	20	20	20	20	20	20
	mean	-35.4	-28.2	5.47	-36.8	-20.9	-20.8
	SD	86.7	67.2	55.3	82.8	75.3	97.2
	median	-7.57	-7.63	21.2	-11.99	-5.96	16.62
Mann-Whitney	U	130	176	212	175	161	206
	z	2.27	1.11	0.20	1.13	1.49	0.37
	P	0.023	NS	NS	NS	NS	NS

Mann-Whitney U test. 1st Samples: prior to change from BI to HI therapy; 2nd Samples: 3-8 months after transferring to HI therapy. 3rd Samples: >12 months on HI therapy. H=human insulin; P=pork insulin; B=beef insulin at 10 and 100ug.ml^{-1}. SD=standard deviation, z=standard normal deviate, P=probability.

2.4.2.(b) Effects Of Transferring From Beef to Human Insulin Therapy

Table 2.22.(a) and 2.22.(b) and 2.22.(c) examines the statistical difference in SI of the 1st, 2nd and 3rd samples of group II diabetic patients.

According to the results, the *in vitro* lymphocyte proliferation in the presence of beef insulin (and pork insulin - samples 1 and 3) increased when the patients were transferred from beef to human insulin therapy. As discussed earlier, these findings may be due to the distortion in SI values caused by the presence of FCS in culture medium during analysis of the 1st samples. The stimulation index of 2nd and 3rd samples does not significantly differ from each other.

In Table 2.23.(a), 2.23(b), 2.23(c)., the % suppressor cell activity of the three samples are compared with each other.

The results show that there is no significant difference in % suppression between the three samples. There was, however, in many instances a significant correlation in % suppression and SI between the various types of insulin, this is illustrated in Tables 2.24.(a) and 2.24.(b).

Table 2.22.(a)

Difference in SI of 1st and 2nd samples:

		Type & Concentration of Insulin *in vitro*					
		H10	H100	P10	P100	B10	B100
	N	-	-	18	18	18	18
Wilcoxon	T	-	-	55	57	30	40.5
	z	-	-	1.31	1.22	2.4	1.94
(probability)	P	-	-	NS	NS	0.017	0.053*

*H=human insulin; P=pork insulin; B=beef insulin at 10 and 100ug.ml^{-1}. z=standard normal deviate. * The increase in SI just fails to be significant.*

Table 2.22.(b)

Difference in SI of 2nd and 3rd samples:

		Type & Concentration of Insulin *in vitro*					
		H10	H100	P10	P100	B10	B100
	N	18	18	18	18	18	18
Wilcoxon	T	80	61	83	60.5	66.5	65
	z	0.22	1.05	0.09	1.07	0.81	0.87
(probability)	P	NS	NS	NS	NS	NS	NS

H=human insulin; P=pork insulin; B=beef insulin at 10 and 100ug.ml^{-1}. z=standard normal deviate.

Table 2.22.(c)

Difference in stimulation indices of 1st and 3rd samples:

		Type & Concentration of Insulin *in vitro*					
		H10	H100	P10	P100	B10	B100
	N	-	-	18	18	18	18
Wilcoxon	T	-	-	52	25	22.5	51
	z	-	-	1.44	2.61	2.72	1.48
(probablity)	P	-	-	NS	0.009	0.006	NS

Two-sample Wilcoxon test for paired data. H=human insulin; P=pork insulin; B=beef insulin at 10 and 100ug.ml^{-1}. z=standard normal deviate.

Table 2.23.(a)

A comparison of the % suppression of 1st and 2nd samples:

		Insulin Type & Concentration *in vitro*					
		H10	H100	P10	P100	B10	B100
	N	-	-	18	18	18	18
Wilcoxon	T	-	-	51	80	52	51
	z	-	-	1.48	0.22	1.44	1.48
Probability		-	-	NS	NS	NS	NS

Two-sample Wilcoxon test for paired data.
H=human insulin; P=pork insulin; B=beef insulin at 10 and 100ug.ml^{-1}.

Table 2.23.(b)

A comparison of the % suppression of 1st and 2nd samples:

		Type & Concentration of Insulin *in vitro*					
		H10	H100	P10	P100	B10	B100
	N	18	18	18	18	18	18
Wilcoxon	T	76	55	60	74	71	77
	z	0.39	1.31	1.09	0.48	0.61	0.35
Sig. level	P	NS	NS	NS	NS	NS	NS

Two-sample Wilcoxon test for paired data.
H=human insulin; P=pork insulin; B=beef insulin at 10 and 100ug.ml^{-1}.

Table 2.23.(c)

A comparison of the % suppression of 1st and 2nd samplesi:

		Insulin Type & Concentration *in vitro*					
		H10	H100	P10	P100	B10	B100
	N	-	-	18	18	18	18
Wilcoxon	T	-	-	85	60	43	75
	z	-	-	0	1.09	1.83	0.44
Sig. level	P	-	-	NS	NS	0.067*	NS

*Two-sample Wilcoxon test for paired data. The probability (P) values are given for a two-tailed test. *P=0.0337 for a one-tailed test; thefore, the % suppression of 1st samples are significantly lower than that of the 3rd sample. H=human insulin; P=pork insulin; B=beef insulin at 10 and 100ug.ml^{-1}.*

Table 2.24.(a)

Spearman's Rank Correlation Coefficient of SI and % suppression between the three types of insulin. Value of (r_s): Group II patients - **2nd sample.**

			Stimulation Index (SI)					
			H10	H100	P10	P100	B10	B100
	H10	N=			18		18	
%		r=			0.511*		0.392	
S	H100	N=				18		18
U		r=				0.575*		0.402
P								
P	P10	N=	18				18	
R		r=	0.699**				0.796***	
E								
S	P100	N=		18				18
S		r=		0.276^{NS}				0.639**
I								
O	B10	N=	18		18			
N		r=	0.629**		0.8***			
	B100	N=		18		18		
		r=		0.465		0.585*		

**=P<0.05*
***=P<0.01*
****=P<0.001*
Top right triangle show the correlation in SI obtained with H=human; P=pork; and B=beef insulins at 10 and 100ug.ml^{-1}. Bottom left triangle gives the correlation in % Suppression obtained with the three types of insulin in culture.

Table 2.24.(b)

Spearman's Rank correlation coefficient of SI and % suppression between the three types of insulin. Value of (r_s): Group II patients -**3rd sample.**

			Stimulation Index (SI)					
			H10	H100	P10	P100	B10	B100
	H10	N=			18		18	
%		r=			0.282		0.738***	
S	H100	N=				18		18
U		r=				0.498*		0.493*
P								
P	P10	N=	18				18	
R		r=	0.307				0.189	
E								
S	P100	N=		18				18
S		r=		0.422				0.556*
I								
O	B10	N=	18		18			
N		r=	0.649**		0.15			
	B100	N=		18		18		
		r=		0.827***		0.556*		

**=P<0.05*
***=P<0.01*
****=P<0.001*
Top right triangle show the correlation in SI obtained with H=human; P=pork; and B=beef insulins. Bottom left triangle gives the correlation in % Suppression obtained with the three types of insulin in culture.

Analysis of data from the first samples showed no correlation between the response (SI) to beef and pork insulins (results not shown). There was however, a significant correlation in the % suppression between pork and beef insulins at 10 ug.ml^{-1} (r=0.47; N=18; P<0.05).

The correlation coefficient values for the response to human, pork and beef insulins of the 2nd and 3rd samples are tabulated in Tables 2.24.(a) and 2.24.(b) respectively. Table 2.24.(a) shows that in the 2nd

samples there was a significant correlation in SI between pork and human insulins, with stronger correlation between the responses to beef and pork insulins. The response to beef and human insulins neither differed significantly nor correlated with each other. The data on % suppression however, showed significant correlation between all three types of insulin. In Table 2.24.(b) it can be seen that for the 3rd sample a significant correlation in SI to all three types of insulin exists (at least at one concentration level). There was a strong correlation between the % suppression with beef and human insulin as well as with beef and pork insulins, but not with human and pork insulin.

2.5. DISCUSSION

The present investigation demonstrates that lymphocytes from both non-diabetic control subjects and diabetic patients, proliferate *in vitro* in the presence of pork, beef and human insulins. Moreover, the lymphocyte proliferative response of Group I diabetic patients to human insulin does not significantly differ from that of control subjects, implying that T-cells from diabetics and non-diabetics probably recognize the same epitopes on human insulin but not on beef and pork insulins: diabetics gave significantly higher SI values to pork ($P<0.01$) and to beef ($P<0.02$) insulins.

Lymphoid cells have two distinct insulin receptors, the immunological receptor on insulin specific B- and T-cells and the hormonal receptor on most activated lymphocytes. It is unlikely that the responses are due to the stimulation of the hormonal receptor since the insulin concentrations (10-100ug.ml^{-1}) employed in the culture system are in vast excess of the maximum concentration for physiological hormonal responses (10^{-8}M) (Blundell et. al. 1972). Also, the concentration of U-100 insulin administered therapeutically to the diabetic patients, is 3.85 mg.ml^{-1}. Therefore, lymphocytes from diabetic patients are most likely exposed *in vivo* to insulin concentrations in the range that is optimal for

in vitro responses. The insulin preparations used in culture had very little contamination; proinsulin (<0.001%, none in HI) and glucagon (<0.001%, none in HI). Therefore, these contaminants are unlikely to stimulate the lymphocytes. The frequency of non-responsiveness among diabetics (60%) and controls (82%), also make hormonal effects an unlikely explanation for the responses observed.

The fact that both insulin treated diabetic patients and control subjects possess T-cells that are autoreactive to human insulin suggests that T-cell autoreactivity to human insulin does not necessarily relate to the onset of type I diabetes as has been suggested by several papers (MacCuish et. al., 1975; Miller et. al., 1987). A T-cell response to human insulin may reflect anti-self immune responses arising in normal and diabetic individuals from pancreatic B-cell autolysis (Naquet et. al., 1988). Intercellular self proteins are thought to be routinely released from lysed cells into the blood circulation and might in fact be presented to T-cells by a variety of cell types in association with self MHC antigen (Clavertie, et. al., 1986). Such autoreactive T-cell responses would not lead to autoimmune disease if they were suitably immunoregulated. Similarly, autoimmunity to human insulin may stem from recognition of "self" epitopes by cells that escape normal immune regulation or tolerance mechanisms (Naquet et. al., 1988). Similar results are reported in mice (Jensen &

Kapp, 1985), i.e. mice react to autologous insulin.

There was a strong correlation in response to all three types of insulins in both groups of diabetic patients, especially in the presence of high insulin concentration (100 $ug.ml^{-1}$). Insulin is known to form dimers and hexamers at high concentrations (>30$ug.ml^{-1}$) (Kontiainen, 1982), and cells from diabetic patients may react to such complexes formed *in vitro* by all three types of insulin. The insulin used in the assay contains between 0.4% to 0.9% zinc. Zinc is also known to induce insulin to form complexes which results in conformational changes on the surface of the molecule. These changes, which principally involve the shift of amino acid side chains out to the surface of the molecule may also exist in the monomer (Nell et. al., 1985). Because therapeutic insulin preparations contain concentrations that favour the formation of complexes including dimers, hexamers and larger aggregates, all insulin-treated diabetic patients have been exposed to determinants present on these complexes. The *in vitro* response probably reflects recognition of such determinants. Also, because the zinc-dependent alterations arise at sites not influenced by amino acid sequence variation between species, they may explain some shared reactivities to human and animal insulins.

Interestingly, of the four patients who were on zinc-insulin therapy alone (Human Ultratard - crystalline

insulin-zinc suspension), two gave strong proliferative responses to all three types of insulins, one patient gave strong responses to pork and beef insulins (his cells were not assayed with human insulin) and the fourth patient (No.30) gave poor response to all three types of insulins, (Table 2.25.). The strong responses *in vitro* to the three insulins may be due to zinc-insulin therapy.

Table 2.25.

Lymphocyte proliferative response (SI) to human , pork and beef insulins by patients on zinc insulin therapy:

Patient Number	Type & Concentration of Insulin H10	H100	P10	P100	B10	B100
11	-	-	1.66	2.47	1.79	2.12
30	0.66	0.65	0.76	0.43	0.75	0.76
49	1.04	2.91	1.25	2.75	1.02	2.85
51	2.14	2.35	2.01	1.71	0.95	3.29

Stimulation Index at 10 and 100 ug.ml^{-1} of H=human insulin; P=pork insulin ; and B=beef insulin.

Another explanation for the high correlation in the responses may be that cells from diabetic patients and control subjects recognize epitopes that are common to all three species of insulin due to the high degree of amino acid sequence homology. For example, Nell et. al., (1985) found that some subjects require the serine residue at position A9 for optimal responses to low concentrations of beef insulin. This residue is also present in human and pork insulins. In mice, the hydroxyl group of this serine residue has been shown to

influence the immunogenic determinants recognized by some mice; in humans it may be recognized by autoreactive T-cells.

One patient of particular interest was patient number 34 (see Table 2.8) because of his known allergic reaction to insulin at the injection site. He was a 16 year old boy with poor diabetic control (HbA1= 16%) and high insulin dose requirement (1.6 U/day/Kg). He has been an insulin-dependent diabetic for over 14 years and has been on human insulin (Human Isophane (prb)) therapy for 2 years. His cells gave a positive proliferative response to all three types of insulin with stronger responses to beef and pork insulins: maximum SI with HI=2.79; PI=4.24; and BI=4.36. The corresponding % suppression values were: -4.5% in the presence of HI;-38% with PI; and -7% with BI. Furthermore his cells showed a dose response effect with all three types of insulin. A blood sample from this subject was taken on one other occasion and a dose response assay showed similar results ie. an increase in insulin concentration in culture was associated with an increase in SI. The inverse was true for the % suppression, when it appeared that at low concentrations of insulin, the proliferative response was suppressed by 56.6% and 32.9% in the presence of human and pork insulins respectively. It would seem also that his $OKT8^+$ cells failed to recognise beef insulin, since negative % suppression values were observed at both

concentrations used (10 and 100 ug.ml^{-1} of BI). It is possible that this patient's suppressor cells recognize epitopes on the A-chain (A 8-10) where beef insulin differs from pork and human insulins by two amino acid exchanges. Moreover, the failure of these OKT8^{+} cells to function effectively at high concentrations of insulin may contribute to hypersensitivity experienced by this subject at the injection site where the insulin is introduced at high concentrations.

The three patients who were known to show insulin resistance (Nos. 1, 40 and 73) did not give a positive response with all three types of insulins.

Only one patient (No. 29) responded exclusively to human insulin (Table 2.8). One patient (No. 56) responded exclusively to pork insulin and three patients (Nos. 43, 52 and 53) responded solely to beef insulin. Patients 1 to 21 are not included in this discussion because their response to human insulin was not determined. All other patients gave variable degrees of response to at least two types of insulin.

These results not only reflect the complexity of the immune response to insulin but also the diversity in genetic background of the outbred human population (Scheinin et. al., 1983; Mann et. al., 1983; Naquet et. al., 1988; Miller et. al., 1987). The immune response to insulin is under Ir gene control. For example, HLA-DR4 is reported to be associated with response to beef

insulin and HLA-DR3 is associated with responses to beef and pork insulins (Mann et. al., 1983). Therefore, the responses outlined in Table 2.8. may partly reflect the distribution of HLA genotype among the diabetic patients.

For many years, the high response to beef and pork insulins was thought to be due mainly to the difference in amino acid composition of the B-chain of these two types of insulin compared to autologous insulin. However, since then significant responses to therapeutic human insulin (which is identical to autologous insulin in terms of amino acid sequence) has been reported even in patients who have never been exposed to non-human insulins, (Naquet et. al., 1988; Parker & Reeves, 1989). Therefore, the results tabulated in Table 3.10. confirm the hypothesis that the primary structure of the insulin molecule is not the only factor determining its immunogenicity.

Other factors affecting the cellular immune response to insulin include the preferential recognition of tertiary structures by T-cells. For example, Naquet et. al., (1988) showed that the epitope(s) recognized by T-cells require interaction between several residues present on both the A- and the B-chains rather than just the single amino acid exchange. The way the antigen presenting cells process the insulin molecule may also make it more immunogenic. It has been demonstrated that accessory cells can take up and degrade antigen by a metabolically

active process that requires lysosomal enzymes (Unanue et. al., 1984). The degraded antigen (potentially in the form of peptides, may not possess the same epitopes present on the native protein), is then re-presented on the accessory cell surface in association with Ia antigens for recognition by T-cells. Similarly, it is possible that all three insulin molecules are processed by antigen presenting cells in such a way that immunogenic determinants are created which are not present in the native molecules.

Regulation of the immune response to insulin at the cellular level.

The insulin-specific suppressor cell activity of diabetic patients did not differ significantly from control subjects. Diabetic patients, referred to in this study, have long established insulin dependency. Suppressor cell activity of such patients is also reported by others (Buschard, et. al., 1982) not to be significantly different from control subjects. It should be noted however, that the suppressor cell activity measured by Buschard et. al., (1982) was not insulin-specific. The patients' cells were stimulated non-specifically with Con. A and the inhibitory effect of these cells were assessed by culturing with allogeneic lymphocytes, also stimulated by Con. A.

The majority of the Group I diabetic patients, in this

study, gave strongest proliferative responses to beef and pork insulins, yet these patients have been on human insulin therapy for at least 6 months and most of them have never been exposed to beef insulin. This type of heteroclytic response has also been reported by other workers (Parker & Reeves, 1989; Naquet et. al., 1989; Jensen & Kapp, 1985), and may reflect immune regulation at the cellular level. Of the 12 patients who gave maximum response to beef insulin (Figure 2.7.(c)) 9 lacked suppressor cell activity to BI. 5 of 8 patients who gave a maximum response to pork insulin, gave negative % suppression values. It is possible that in these patients, the *in vitro* response to pork and beef is regulated by cells activated by suppressor epitope(s) present on human insulin. If such an epitope is located within the B-chain (B-30 of HI differs from PI and BI) then the human-insulin specific T-suppressor cells would fail to recognize beef and pork insulins *in vitro*, thus producing a heteroclytic response.

Kontiainen (1982), also concluded that the determinants recognized by suppressor cells may be in the B-chain since beef insulin suppressor factor of H-2^b mice was not absorbed by beef A-chain immunoabsorbents in his studies. Alternatively, there may well be multiple partially overlapping suppressor cell inducing determinants in the insulin molecule, which is induced by the physicochemical form of insulin i.e. insulin is mainly monomeric at

concentration <1ug.ml^{-1} and mainly hexameric at doses >30 ug.ml^{-1}, the presence of zinc also induces formation of complexes. Recognition of such overlapping determinants and complexes by cells may explain why some of the patients also showed suppression against pork and beef insulins.

The evidence for the role of $CD8^+$ cells in the regulation of the immune response to insulin is further substantiated by the finding that among Group I diabetic patients, there were 45 positive SI responses (Table 2.8.), 33 (73%) of which were accompanied by negative % suppression values (Table 2.11). Furthermore, a strong negative correlation between % suppression and stimulation index was observed - showing that an increase in % suppression is associated with a decrease in stimulation index. This is shown in Table 2.26.

Table 2.26.

Relationship between Stimulation Index and % Suppression.

	Type & Concentration of Insulin					
	H10	H100*	P10	P100	B10	B100
N	39	39	59	61	60	61
r_s	-0.4002	-0.528*	-0.397	-0.341	-0.605	-0.412
P	<0.05	<0.001*	<0.01	<0.01	<0.001	<0.01

*r_s=Spearman's Rank Correlation Coefficient. *Product moment correlation coefficient. H=human insulin; P=pork insulin; B=beef insulin, at 10 and 100ug.ml^{-1}. N=number of patients. P=probability, significance level.*

Group I diabetic patients were found to exhibit a decrease in % suppression with increase in age ($r=-0.36$; $P<0.05$). This may be a very important finding in view of the importance of suppressor cells. The immune system is a fine balance between effector and suppressor elements, and immunological tolerance is mediated via the generation of a population of suppressor cells. If this delicate balance malfunctions in any way, then an excess of antibodies as in allergic reactions, or the production of aberrant antibodies such as in autoimmune disorders might prevail. Alternatively, there could be a lack of antibodies which might result in immunodeficiencies (Marx, 1975).

Many investigators consider that suppressor T-cells normally prevent the production of auto-antibodies and a deficiency may contribute to autoimmunity. This may explain why diabetic patients experience more immunological complications with age, and the progression from non-insulin dependency to insulin dependency may be due to lack of suppressor cells which would normally reduce the autoimmune process. This view is supported by Marx (1975) who found that in NZB mice there is a decrease in suppressor cell activity with age, which is accompanied by the spontaneous developement of a condition similar to systemic lupus erythematosus in man. The cause of the loss of suppressor T-cells is unknown, but viruses and genetic factors probably play a role (Marx, 1975). It is suggested that a decline in T-cells

and T-cell functions, particularly suppressor T-cells in conjunction with an increase in B-cells, might be correlated to the known increased incidence of autoantibodies with old age (Williams & Messner, 1975).

The relationship between % suppression (in the presence of 10ug.ml^{-1} human insulin) and the patients' daily insulin dose is less easily explained. The rise in % suppression with increase in insulin dose is probably due to suppressor T-cells being activated *in vivo* in patients requiring higher insulin doses. These suppressor cells exert their function *in vitro* when cultured in the presence of human insulin but not in the presence of beef and pork insulins. This is probably because the suppressor cells are human insulin specific.

The results outlined in the present study show that patients with poor diabetic control gave higher insulin-specific stimulation index values than those with good diabetic control ($P<0.05$). Glycosylated haemoglobin levels are an indication of diabetic control over the previous 4-6 weeks prior to the time blood samples were taken from the patients. It is possible that patients with poor diabetic control are under insulinized. ie. there may be an absolutely reduced level of insulin, or at least a relatively reduced insulin level insufficient to maintain glucose at normal levels. In the obese and non-insulin dependent patients who have come to need insulin therapy there is clear evidence for insulin

resistance (Irvine, 1980). The high stimulation index values by patients with poor diabetic control may be associated with such relative or absolute insulin resistance.

Effects Of Transferring From Beef To Human Insulin Therapy On The Cellular Immune Response To Insulin.

It is thought that therapeutically administered biosynthetic and semisynthetic human insulin preparations are less immunogenic (than beef or pork insulins) because of the 100 percent amino acid sequence homology with autologous insulin. Thus, one would expect immunological complications such as insulin resistance, lipoatrophy and hypersensitivity of the delayed type to decrease if patients are transferred to human insulin therapy. This would also be reflected by a decrease in insulin-stimulated proliferation when immune cells of such patients are challenged with insulin *in vitro*.

The above hypothesis is refuted by the results obtained in the present study. For example, the *in vitro* lymphocyte proliferation to 10 $ug.ml^{-1}$ beef insulin increased when the patients were transferred from beef to human insulin therapy.

This was not observed at 100$ug.ml^{-1}$. There was also a significant increase in response between the first and

third sample at 100ug.ml^{-1} pork insulin. The cellular immune response to human insulin was not determined while the patients were on beef insulin therapy. Therefore, it is not possible to comment on the possible effects of transferring from beef to human insulin therapy on the *in vitro* response to human insulin.

The low response of the first samples (due to the use of FCS in culture media) may be responsible for the 'apparent' increase in SI of the second and third samples. There was however, little evidence to suggest that therapeutically administered highly purified beef insulin is more immunogenic than human insulin as has been suggested by many workers (Mann et. al., 1983; MacCuish et. al., 1975). The higher SI values obtained in patients undergoing beef insulin therapy by some workers may be due to factors other than just amino acid sequence difference. There is much evidence to suggest that the pH, purity, solubility and zinc concentration of the insulin preparations as well as the mode of administration, all contribute towards its immunogenicity (Reeves, 1980, 1986). The results outlined in this study suggest that the proliferative response to beef insulin is stronger compared to human insulin, but this was not statistically significant.

It is possible to explain, in part, the proliferative response to beef and human insulins in terms of immunoregulation at the cellular level. For example, the

% suppression in the presence of beef insulin decreases ($P<0.05$) when the patients were transferred to human insulin therapy. It is possible that while the patients are on beef insulin therapy they possess suppressor T-cells that are beef insulin specific (perhaps these cells recognize epitope(s) on the A-chain: A-8 and 10), the response to beef insulin was therefore suppressed. After 3-8 months on human insulin, the number of suppressor cells recognizing beef insulin have probably decreased. The ratio of beef insulin specific suppressor:helper cells also decreased thus giving rise to a heteroclytic response. ie. the response to beef insulin, which is more foreign, increased when the patients were on human insulin.

CHAPTER III

3. *HUMORAL IMMUNE RESPONSE TO INSULIN.*

3.1. INTRODUCTION

In man, the immune response to insulin was first documented by Berson et. al., (1956) who demonstrated the presence of insulin binding immunoglobulins in the serum of diabetic patients treated with beef and pork insulins. Subsequent studies have documented the presence of anti-insulin antibodies in most insulin treated diabetic patients, even in those on human insulin therapy (Andersen, 1972; Asplin et. al., 1978; Di Mario et. al., 1986; Pickup, 1986).

A minority of these patients are reported to develop allergic reactions and immunological complications which may result in asthma, urticaria and anaphylactic shock. The presence of very high concentrations of insulin antibodies is known to cause insulin resistance with subsequent effects on the dose requirements and pharmaco-kinetics of the injected insulin (Caugahan, et. al., 1983). Recent studies, however, suggest that moderate concentrations of insulin antibodies are not responsible for insulin resistance and may in fact improve diabetic control by providing a buffering system capable of maintaining a more steady basal level of free insulin concentration (Gray, et. al., 1981; Gray, et.

al., 1985). In patients with vascular disease, however, insulin antibody complexes may have a deleterious effect (Andersen, 1976, Reeves, 1980; 1986).

Insulin antibody formation depends on a variety of factors, including the pattern of insulin therapy (i.e. single or multiple injections per day), mode of administration, pH, zinc and protamine content, degree of purity and species of origin. For example, beef insulin is said to be more immunogenic than pork or human insulins, and highly purified insulins induce lower insulin antibodies than conventional preparations (Reeves, 1980; Wilson, et. al., 1985; Di Mario, et. al., 1986; Pickup, 1986). The physical state of the insulin preparation, irrespective of the species of origin, can also influence the degree of anti-insulin antibodies produced. Patients who require soluble insulin in addition to isophane are reported to show a significant enhancement of antibody production (Reeves, et. al., 1984), and contaminants such as zinc are well documented to produce increased levels of antibody (Lunetta, et. al., 1986).

Many studies have correlated the anti-insulin antibody level in insulin dependent diabetic patients with certain HLA antigens (Schernthaner, 1982; Schernthaner, et. al., 1979; MacEvoy, et. al., 1986; Almer, et. al., 1985; Asplin, et. al., 1984; Karjalainen, at. al., 1989). Nearly all of these studies report a lower incidence of

anti-insulin antibody in patients possessing HLA-DR3 and HLA-B8 whereas some workers have shown that the level of antibody is increased in patients with HLA-DR7 antigen.

Sklenar et. al., (1982) have found that diabetic patients who are DR3 carriers have a low responder status to pork but not to beef insulin. DR4 carriers and DR4/DR3 heterozygotes, on the other hand, show a high responder status to beef as well as to pork insulins. The high responder status of the DR4/DR3 carriers to pork insulin suggests that the gene(s) associated with HLA-DR4 (and responsible for a high response to pork insulin) is (are) dominant to gene(s) associated with HLA-DR3 (and a low response) with regard to the production of antibodies to pork insulin.

Recently, the level of insulin antibody production has been reported to be influenced by a gene(s) linked to the IgG heavy chain complex (Gm allotype), and not to the MHC (Nakao, et. al., 1981; Reeves et. al., 1984). These conflicting results undoubtedly reflect the outbred nature of the human population and the complexicity of the genetic regulation of the immune system.

All five major classes of immunoglobulins (G,M,A,E and D) have been found in insulin-treated diabetic patients (Page-Faulk, et. al., 1971); quantitatively, the most significant antibodies are those of the IgG class. Most studies, investigating the humoral immune response to

insulin, have only concentrated on the level of total IgG anti-insulin antibody. Few, if any, have looked at the anti-insulin IgG subclasses. Yet, animal experiments suggest that the subclass of IgG may play an important role in determining the rate of immune complex clearence *in vivo*. In the guinea pig, for example, two anti-insulin IgG subclasses can be identified and purified (Yagi, et. al., 1962), IgG1, which does not activate complement and IgG2, which binds C1q and specific Fc receptors on macrophages.

Since the physicochemical and biological properties of the four human IgG isotypes are known to be different (Shakib, 1986), the class and subclass of anti-insulin immunoglobulins could be of great clinical significance. Koch , et. al., (1986) have made a preliminary investigation, but the number of patients used were limited (24 patients) and they failed to look at the possible relationship between IgG subclass and clinical background of the diabetic patients.

In the present study, the class of immunoglobulin produced by diabetic patients (IgM and IgE), the level of anti-insulin IgG and IgG subclasses were determined in an attempt to investigate their possible clinical relevance. The effect of beef insulin and human insulin therapy on the level and type of anti-insulin antibody produced was also compared.

3.2. MATERIALS AND REAGENTS

(i) Serum samples : 5 to 10 ml blood samples were collected from Type I Diabetic patients and control subjects. The blood was allowed to clot at 4°C for several hours before being spun down at 400 x g for 10 minutes. The Serum layer was recovered and aliquoted into glass tubes and stored at -20°C until further use.

(ii) Standard and Quality Control (QC) serum samples were kindly donated by Dr. T.J. Wilkin and Miss A. Tuck of Southampton General Hospital, Southampton.

(iii) *Conjugate 1.* (GAHu/IgG(H+L)/PO : Goat anti-human IgG (Fc + Fab) conjugated to Horseradish Peroxidase - affinity purified (Nordic Immunological laboratories, Madienhead, Berkshire). Diluted 1 in 10 with sterile d.d. water and stored at 4°C. The conjugate was further diluted 1 in 200 with PBS-Tween 20 (pH 7.2) to give a working dilution of 1 in 2000.

(iv) *Conjugate 2.* (GAM/IgG/PO : Goat anti-mouse IgG labeled with Horseradish Peroxidase - (Nordic Immunological laboratories, Madienhead, Berkshire, Cat. No. 04.18.02).

(v) *Conjugate 3.* (GAHu/IgE(Fc)/PO) : Goat anti-human/IgE(Fc specific) conjugated to Horseradish Peroxidase, 1 ml lyof. reconstituted with 1 ml d.d. water - (Nordic Immunological laboratories, Madienhead, Berkshire)

(vi) Mouse monoclonal antibodies to humun IgG subclasses:

IgG subclass	*Cat. No.*	*WHO/IUIS study code*	*Clone No.*
Anti-IgG1 -	M15015	HP6012	NL16
Anti-IgG2 -	M14017	HP6009	GOM2
Anti-IgG2 -	(from Sigma)	HP6014*	
Anti-IgG3 -	M08010	HP6010	ZG4
Anti-IgG4 -	M11013	HP6011	RJ4

All anti-IgG subclasses, except *, were obtained from Oxoid Ltd., Bassingstoke, England. The above monoclonal antibodies (Mab) were derived from Murine hybridomas.

(vi) Anti-IgM, code No. M02012, clone No. AF6 (Oxoid Ltd.).

(vii) Human Immunoglobulin G standard (10 mgs ml^{-1}), (Sigma Chem Co. St. Louis MO).

(viii) Human Immunoglobulin E (0.1 mg ml^{-1}) (Serotec Ltd., Blackthorn, Bicester, England).

(ix) Insulin : Novo human (monocomponent (mc), biosynthetic), pork (mc) and beef (mc) were obtained on prescription from the Local Chemist. The Insulins came as 100 u (3.85 mg ml^{-1}) clear solutions, which were made up in carbonate/bicarbonate buffer pH 9.6.

(x) CNBr-activated Sepharose 4B beads, freeze dried

(Pharmacia LKB Biotechnology, England. Code No. 17-0430-01).

(xi) 1-Component ABTS substrate : Containing buffered 2.2`-azino-di [3-ethyl-benzthiazoline sulphate (6)] (ABTS), and hydrogen peroxide stabilized in one solution (Kirkegaard & Perry Ltd. Billinghurst, Cat. No. 50.66.00).

(xii) Carbonate/bicarbonate buffer, pH 9.6 : 1.5 x 10^{-2} M sodium carbonate (Na_2CO_3); and 3.49 x 10^{-2} M sodium bicarbonate ($NaHCO_3$).

(xiii) Phosphate Buffered Saline (PBS) with Tween-20, pH 7.2 : 0.14 M sodium chloride; 2.68 x 10^{-3} M potassium chloride; 8.1 x 10^{-3} M disodium hydrogen phosphate (anhydrous salt); 1.5 x 10^{-4} M potassium di-hydrogen orthophosphate and 0.05% v/v Tween-20, made up with deionized distilled (d.d.) water.

(xiv) 1 % Sodium dodecyl sulphate (SDS)

(xv) 1 x 10^{-3} M Hydrochloric acid.

(xvi) Coupling buffer, pH 8.9 : 0.1 M Sodium hydrogen carbonate containing 0.5 M sodium chloride.

(xvii) Acetate buffer, 0.1 M Sodium acetate containing 0.5 M sodium chloride.

(xviii) 1 M Ethanolamine in coupling buffer pH 8.0.

(xix) Tris buffer, pH 8.0 : 0.1 M Tris HCl containing 0.5 M sodium chloride.

(xx) 1% BSA and 1% BSA made up in 0.01% Sodium ethylmercuri-thiosalicylate (thimerosal).

(xxi) KCNS Ammonia : 1 M Potassium thiocyanate, 0.5 M Ammonium hydroxide.

(xxii) 96-well flat-bottomed Nunc Immunoplate II. Cat. No. 4-42404 (Gibco laboratories, uxbridge, middlesex).

(xxiii) Skatron A/S Microwash II, automatic plate washer.

(xxiv) M600 Microplate Reader (Dynatech Laboratories Ltd., Billingshurst, Sussex.

(xxv) 5412 Eppendorf Microfuge.

(xxvi) Eppendorf microcentrifuge tubes, 1.5ml volume.

(xxvii) Scintered glass funnel (code No. SF4B32 - Quickfit, England).

(xxviii) Dialysing tubing, 18/32 of an inch, from Scientific Instruments Centre Ltd., Hampshire, England.

(xxix) LKB 2138 UVICORD - UV chart recorder.

(xxx) Philips PU 8800 UV/VIS Spectrophotometer.

3.3. METHODS

3.3.1. Enzyme Linked Immunosorbent Assay (ELISA) Determination of Serum Anti-Insulin Antibody Titer.

Monocomponent human, beef or pork insulin was adhered to 96-well flat-bottomed microtiter plates as follows :

1.5 ug well^{-1} (100 ul of 15 ug ml^{-1}) freshly prepared insulin solution made-up in carbonate/bicarbonate buffer (pH 9.6) was added and incubated at 4°C overnight or at room temperature for 3 hours.

Unbound insulin was removed by washing the plate with PBS-Tween 20 using an automatic Skatron A/S microwash II. Each washing step consisted of five cycles.

In each insulin coated plate, serial dilutions (100ul well^{-1}) of a serum (with known concentration of anti-insulin antibody content) were added as standards. Test serum samples were used at 1 in 30 dilution in PBS-Tween 20 (100ul well^{-1} in duplicate). Following 1 hour's incubation at room temperature the plate was washed. 100 ul of 1 in 2000 diluted, goat anti-human IgG (H+L) conjugated to peroxidase (GAHu/IgG(H+L)/PO), was added to each well and incubated for a further 1 hour at room temperature. The plate was washed again to remove excess conjugate.

100 ul well^{-1} of 1-component ABTS substrate was added. The enzyme-substrate reaction was allowed to proceed until a O.D. $_{410nm}$ of 1.7 for the highest concentration of standard was reached. The reaction was stopped with 100 ul well^{-1} 1% sodium dodecyl sulphate (SDS).

The light absorbance reading was made on a Dynatech MR 600 Microplate reader using a sample wavelength of 410nm and reference wavelength of 490nm. The O.D. for threshold level was set at 1.99 and calibration value was set at 1.00. The plate reader was blanked in a well which had recieved all reagents but no test sample.

Note. The outer wells of the Nunc 96-well microtiter plate were not used for sample analysis because variation in signal strength was sometimes observed in these wells, i.e. there was an "edge effect".

3.3.2. ELISA - IgG Subclass Determination.

Micro plates were coated with 15 ug ml^{-1} human (or beef) insulin made up in carbonate/bicarbonate buffer (pH 9.6). The plates were incubated at 4°C overnight. Between each stage of the assay the plates were washed as described previously.

Samples (100ug well^{-1}) were added at a dilution of 1 in 30 :

Each plate was divided into four equal segments so that each sample was tested in duplicate in each segment, as shown below.

Layout of serum samples and anti-IgG subclasses on a 96 well microtiter plate:

	IgG1						*IgG2*					
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A												
B		1	2	3	4	5	1	2	3	4	5	
C		1	2	3	4	5	1	2	3	4	5	
D		Neg	Neg	Std	Std		Neg	Neg	Std	Std		
E		1	2	3	4	5	1	2	3	4	5	
F		1	2	3	4	5	1	2	3	4	5	
G		Neg	Neg	Std	Std		Neg	Neg	Std	Std		
H												
	IgG3						*IgG4*					

Neg.=negative control;
Std.=standard (positive) control.
Numbers 1 to 5 are test serum samples.

Following 1 hour´s incubation at room temperature, the plates were washed. Into the wells of each segment of the plate, 100ul of a different mouse anti-IgG subclass (diluted 1 in 500) was added so that each sample was tested for the presence of all four anti-insulin IgG

subclasses on the same plate.

After 1 hour at room temperature, the plates were washed and 100 ul of 1 in 1000 diluted, goat anti-mouse Ig conjugated to horseradish peroxidase (GAM/Ig(Y)/PO) was added to each well. Following a further hour's incubation at room temperature, the plates were washed with PBS-Tween 20. 100ul of 1-component ABTS-substrate was added to each well. The enzyme-substrate reaction was allowed to proceed until a maximum O.D. (at $_{410nm}$) of 1.7 was reached. The reaction of each sample was stopped separately with 100ul well^{-1} 1% SDS.

3.3.3. *Coupling of Insulin to CNBr-activated Sepharose4B beads*

Two grams of freeze dried CNBr-activated Sepharose 4B beads was suspended in 1 mM HCl (each gram of beads gave approximately 3.5 mls of swollen gel). The gel was washed with 1 mM HCl for 15 minutes on a scintered glass filter. Approximately 200 mls of 1 mM HCl was used to wash each gram of freeze dried beads. The ligand (human insulin or 1% BSA) was diluted in coupling buffer pH 8.9 (0.1 M $NaHCO_3$ containing 0.5 M NaCl) at 1.155 mg ml^{-1}. The gel was transferred to 15 mls of ligand solution and mixed by end-over-end rotation for 2 hours at room temperature.

Excess ligand was washed away with coupling buffer and any remaining active groups on the beads were blocked with 1 M ethanolamine (pH 9.0), for at least 4 hours at 4°C. The product was washed with three cycles of alternate pH. Each cycle consisted of a wash with 0.1 M acetate buffer (pH 4.0) containing 0.5 M NaCl, followed by a wash with 0.1 M Tris buffer (pH 8.0) containing 0.5 M NaCl. The resulting product was stored at 4°C as a 50% slurry in PBS/0.1% sodium ethylmercuri-thiosalicylate (thimerosal).

Absorbance reading of insulin solution before coupling :

$$_{280nm} = 2.3 \qquad (1.155 \text{ mg ml}^{-1})$$

Absorbance reading after coupling to Sepharose 4B beads :

$$_{280nm} = 0.288$$

Therefore, % of insulin absorbed by the beads:

$$2.3 - 0.288 = 2.012$$

$$2.012 \; / \; 2.3 \times 100 = 87.48\% \; .$$

Amount of insulin used :

1.155 (mgs ml^{-1}) x 15 (mls) = 17.325 mgs.

87.48 % of 17.325 = 15.155 mgs per 2 gms of beads.

Therefore, 7.57 mgs of insulin is coupled to each gram of Sepharose 4B beads.

3.3.4. *Absorption of Human Serum with Sepharose 4B Beads coupled to Insulin.*

Plastic micro centrifuge tubes were coated with 1 ml of 1% BSA made up in PBS/ethimerosal, by end-over-end rotation overnight at 4°C. This blocks non-specific binding of antibody to the tubes. The tubes were washed 3 times with PBS and dried on paper towels.

100 ul of 50% slurry CNBr-activated Sepharose 4B beads coupled to human insulin was placed into each tube and centrifuged for 1 minute in an 5412 eppendorf microfuge. The supernatant was removed and 270 ul of PBS-Tween 20 was added to each tube. 30 ul of serum sample was also added (this gave a serum dilution of 1 in 10).

The mixture was rotated gently at room temperature for 2 hours. The tubes were centrifuged for 3 minutes and the supernatants (absorbed sera) were harvested to be assayed using the ELISA method outlined in section 3.3.1.

The above procedure was repeated using 50% slurry of CNBr-activated Sepharose 4B beads coupled to 1% BSA in place of insulin coated beads.

Note: The serum is now 1 in 10 diluted. However, 1 in 30 dilution is required for ELISA (see section 3.3.1). Therefore, the above sera were further diluted 1 in 3 with PBS-Tween 20.

3.3.5. *Affinity Purification Of Human Anti-Insulin Antibody*

The anti-insulin antibody titer in the sera of diabetic patients was originally determined in terms of Binding Units (B.U.). Standards obtained from Dr. T.J. Wilkin and others, Southampton General Hospital, were used to determine the 'B.U.' values. Since the expression of antibody titer in terms of binding units was purely arbitrary, quantitation of the anti-insulin antibody in micrograms per ml of serum was necessary.

Serum samples, which gave high binding unit values, were pooled and the anti-insulin antibody was affinity purified on a human insulin conjugated CNBr- activated Sepharose 4B column.

Procedure: affinity purification of anti-insulin antibody.

15.16 mgs of human insulin was coupled to 2 gms of CNBr-activated Sepharose 4B beads as described in section 3.3.3.

A 7 ml column of insulin coupled CNBr-Sepharose 4B beads was prepared and washed through with PBS. The column was pre-eluted with KCNS-Ammonia (1 M potassium thio cyanate, 0.5 M sodium Ammonia) and washed again with PBS. The chromatography was monitered using an UV

detection-chart recorder. 6 mls of pooled sera was diluted with an equal volume of PBS and gently added to the column and allowed to run through. Unbound serum components were washed through with PBS, until the absorbance reading on the chart recorder reached baseline.

The bound insulin antibody was eluted with KCNS-ammonia until the O.D. $_{(280nm)}$ returned to that of KCNS-ammonia (0.5M).

The eluate was collected into a weighed glass vial and the pH was immediately adjusted to 7.3. The resulting affinity purified anti-insulin antibody was placed in a 18/32 inch dialyzing tubing (which had been boiled for two minutes in 0.1% acetic acid to remove unwanted sulphur products). The antibody was dialysed against PBS overnight at 4°C.

The dialysed anti-insulin antibody concentration was determined by spectrophotometric means :

Volume of dialysed antibody = 7.297 mls (7.297 gms)
Absorbance reading $_{280nm}$ = 0.29 Extinction Coefficient for IgG = 14.3 for a 1% solution; 1 cm pathlength.
Concentration (mgs ml^{-1}) = Absorbance $_{280nm}$ / $E^{0.1\%\ 1cm}$.
Therefore, 0.29/1.43 = 0.2028 mg ml^{-1} of serum, or 202.8 ug ml^{-1} of serum.

3.3.6. *Determination Of IgM*

The relative IgM distribution in the sera of diabetic patients and control subjects was determined using an indirect ELISA protocol.

96 well microtiter plates were coated overnight at 4°C with 100ul of 1 in 30 diluted serum in carbonate/bicarbonate buffer (pH 9.6). Into each plate a standard IgM sample, which was known to contain high IgM titer was also assayed as a quality control. The plates were washed with PBS-Tween 20 (five cycles). 100ul of mouse anti-human IgM, diluted 1 in 100 with PBS-Tween 20, was added to each well and the plates incubated at room temperature for 1 hour.

Following five more wash cycles with PBS-Tween 20, 100ul of anti-mouse IgG.HRP (Unipath) diluted 1 in 150, was added to each well and incubated for 1 hour at room temperature.

The plates were washed with PBS-Tween 20, and 100ul of 1-component ABTS-substrate was added. The colour reaction was allowed to proceed until a maximum $O.D._{410nm}$ of 1.7 was reached by the quality control sample. The reaction was then stopped by adding 100ul of 1% SDS to each well, the OD readings were recorded with the plate reader.

3.3.7. *Determination Of IgE In Serum*

The distribution of IgE in serum was determined using a direct ELISA method.

Plates coated with 100ul well^{-1} serum samples (diluted 1 in 30 with carbonate/bicarbonate buffer, pH 9.6) were washed and incubated with 100ul of goat anti-human IgE conjugated to peroxidase (GAH/IgE(Fc)/PO; diluted 1 in 200). Following 1 hour at room temperature, the plates were washed and 100ul of 1-component ABTS-substrate was added to each well. Into each plate, a quality control which was known to have a high IgE titer was placed. The colour reaction was allowed to proceed until a maximum O.D. $_{410nm}$ of 1.7 was reached by the quality control sample. The reaction was then stopped with 100ul of 1% SDS added to each well and the OD readings were recorded with the plate reader.

3.3.8. *Determination of serum Interleukin-2 (IL-2) concentration.*

The concentrations of IL-2 in the sera of diabetic patients and control subjects were determined using a human interleukin-2 intertest-2 kit (Genzyme corporation, Boston). The method was as described by manufacturers. A Polystyrene 96 well plate was coated with mouse monoclonal anti-IL2 antibody which is specific for human IL-2. Following 24 hours incubation at 4°C, the plate

was washed with PBS Tween 20 and specimens and appropriate standard samples were added to the plate and incubated for a further 24 hours at 37°C. Unbound material was removed by aspiration and washing of the wells. The rabbit polyvalent antibody to human IL-2 was then added and incubated for 1 hour at 37°C. Again unbound antibody was removed by washing. The third antibody, an enzyme labelled goat anti-rabbit polyclonal reagent, was incubated in the wells for an hour at 37°C. Unbound third antibody was removed by washing. Bound, immunoreactive IL-2 was quantitated by an enzymatic reaction resulting in a color change by a Dynatech MR 600 microplate reader. The measured absorbance (due to color change) is proportional the the concentration of IL-2 that was present in the original sample.

3.3.9. *Determination of the Optimum Conditions for ELISA*

In order to determine the optimum conditions for the ELISA system, various parameters were analysed. The resulting protocols are shown above (sections 3.3.1. to 3.3.7.).

(a) *Optimal antigen coating of microtiter plates.*

In order to determine the optimum concentration of insulin required for coating, human insulin was titrated across a 96-well flat-bottomed microtiter plate. All other assay conditions were as described in section 3.3.1. The assay was repeated with conjugate (GAHu/IgG(H+L)/PO) diluted 1 in 1000, 1 in 2000, 1 in 4000, and 1 in 8000.

Figure 3.1. illustrates the variation in signal strength with concentration of coating protein (insulin). The absorbance readings obtained with 1 in 1000 diluted GAHu/IgG(H+L)/PO were above the OD maximum (2.0) in the Dynatech MR 600 microplate reader, and therefore, have been omitted. Figure 3.1. shows that between 30 to 15 $ug.ml^{-1}$ insulin the signal strength obtained with all three dilutions of conjugate was relatively constant. However, below 15 $ug.ml^{-1}$ insulin, the signal strength decreases significantly with 1 in 2000 and 1 in 4000 diluted conjugate. Thus 15$ug.ml^{-1}$ insulin was used for

the standard protocol.

Figure 3.1. also shows the effect of conjugate (GAHu/IgG(H+L)/PO) dilution on the signal strength. In the presence of 1 in 1000 diluted conjugate, the O.D. readings were found to reach the O.D. maximum within 2 minutes of adding the substrate. This made 1 in 1000 dilution unsuitable for the assay. 1 in 2000 diluted conjugate was found to give the optimum results at a slower rate (4-5 minutes).

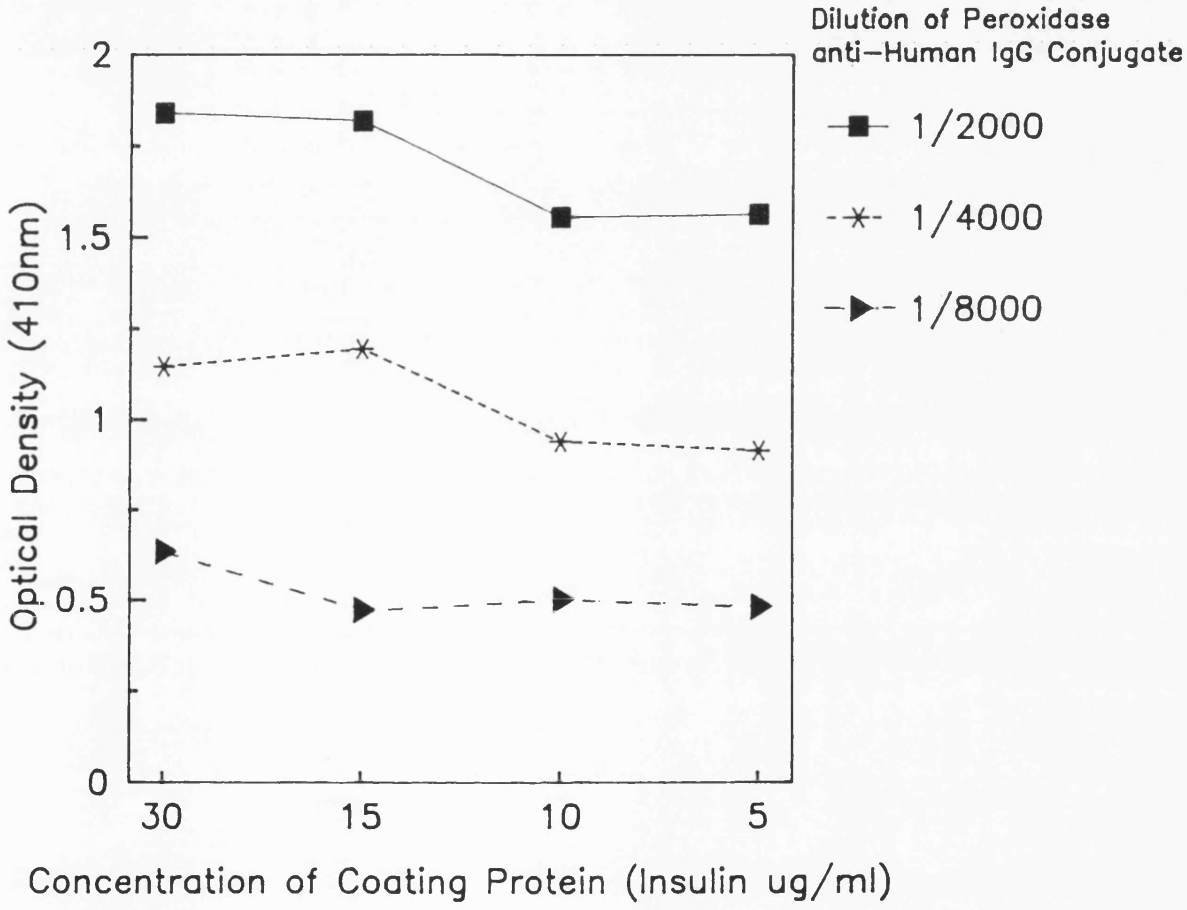
(b) *Absorption and Specificity of Serum Antibody.*

The amount of insulin-coated sepharose 4B beads required to achieve optimum absorption of antibody was determined. Serum from a type I diabetic patient was absorbed with 10-200ul of insulin coated or BSA coated beads (50% slurry). The absorbed serum was analysed using the standardized protocol outlined in section 3.3.1. Table 3.1.(a) shows the effect of varying amount of beads on antibody absorption.

The data for BSA coated beads represents non-specific binding of antibody to BSA, data for insulin coated beads represents the amount of insulin-antibody removed by absorption on the insulin coated beads. Therefore, the difference in absorbance readings of BSA and insulin coated beads is the amount of insulin antibody present in the serum. The data presented in Table 3.1.(a) indicates

Figure 3.1.

Variation in signal strength with concentration of coating protein



that the optimum absorption of insulin antibody is achieved with 100 ul of insulin coated Sepharose 4B beads (50% slurry).

Table 3.1.(a)

Amount of Sepharose 4B beads (50% slurry) required for optimum absorption of insulin antibody.

Volume of beads (ul)	Absorbance (OD) ± SD after absorption with: Insulin coated beads	BSA coated beads	% diff.
200	0.139 ± 0.002	1.255 ± 0.024	88.9%
100	0.142 ± 0.014	1.189 ± 0.005	88.1%
50	0.275 ± 0.004	0.830 ± 0.002	66.9%
25	0.561 ± 0.006	0.931 ± 0.01	39.7%
10	0.789 ± 0.01	0.874 ± 0.002	9.7%

SD=standard deviation. % diff.= % difference in absorbance between insulin coated beads and BSA coated beads, i.e. % antibody absorbed.

In order to determine whether the antibody detected by the assay outlined in section 3.3.1., is insulin specific, serum samples from all subjects (diabetic patients and controls) were absorbed with 100ul (50% slurry) insulin coated or BSA coated beads and reassayed. The procedure is outlined in section 3.3.4. Absorption of antibody with the two types of bead coatings are compared in Table 3.1.(b).

As expected, analysis of negative sera showed little difference in antibody concentration detected in sera absorbed with insulin coated beads and BSA coated beads. Therefore, only positive sera are analysed in Table 3.1.(b).

Table 3.1.(b) shows that the antibody detected is insulin-specific, since absorbtion with insulin coated beads reduced the antibody detected by nearly 80%. There was also some absorption with BSA coated beads (control) which was probably due to non-specific binding.

Table 3.1.(b)

Specificity of Antibody: Positive serum samples only.

	Concentration Of Antibody detected after absorption with: BSA coated beads	Insulin coated beads	% Reduction
N	42	42	42
Minimum	6.7 $ug.ml^{-1}$	0.7 $ug.ml^{-1}$	54.6
Maximum	116.7 $ug.ml^{-1}$	6.4 $ug.ml^{-1}$	97.8
Range	110.0	5.7	43.2
Mean	18.2 $ug.ml^{-1}$	2.8 $ug.ml^{-1}$	79 .0
SD	18.1	1.1	10.2

N=number of samples.
SD=standard deviation.

In Figures 3.2.(a) and (b), monoclonal antibodies for detecting the IgG subclasses are titrated. Figure 3.2.(a) shows the titration of anti-IgG subclasses against normal serum. Sera from healthy control subjects were pooled and diluted 1 in 30 with carbonate/bicarbonate buffer (pH 9.6). The diluted sera was used to coat all 96 wells of a microtiter plate. Mouse monoclonal anti-IgG subclasses were titrated in duplicate across the microtiter plate, all other steps were as described in section 3.3.2. This gave the distribution of IgG subclasses in normal sera.

Figure 3.2(a).

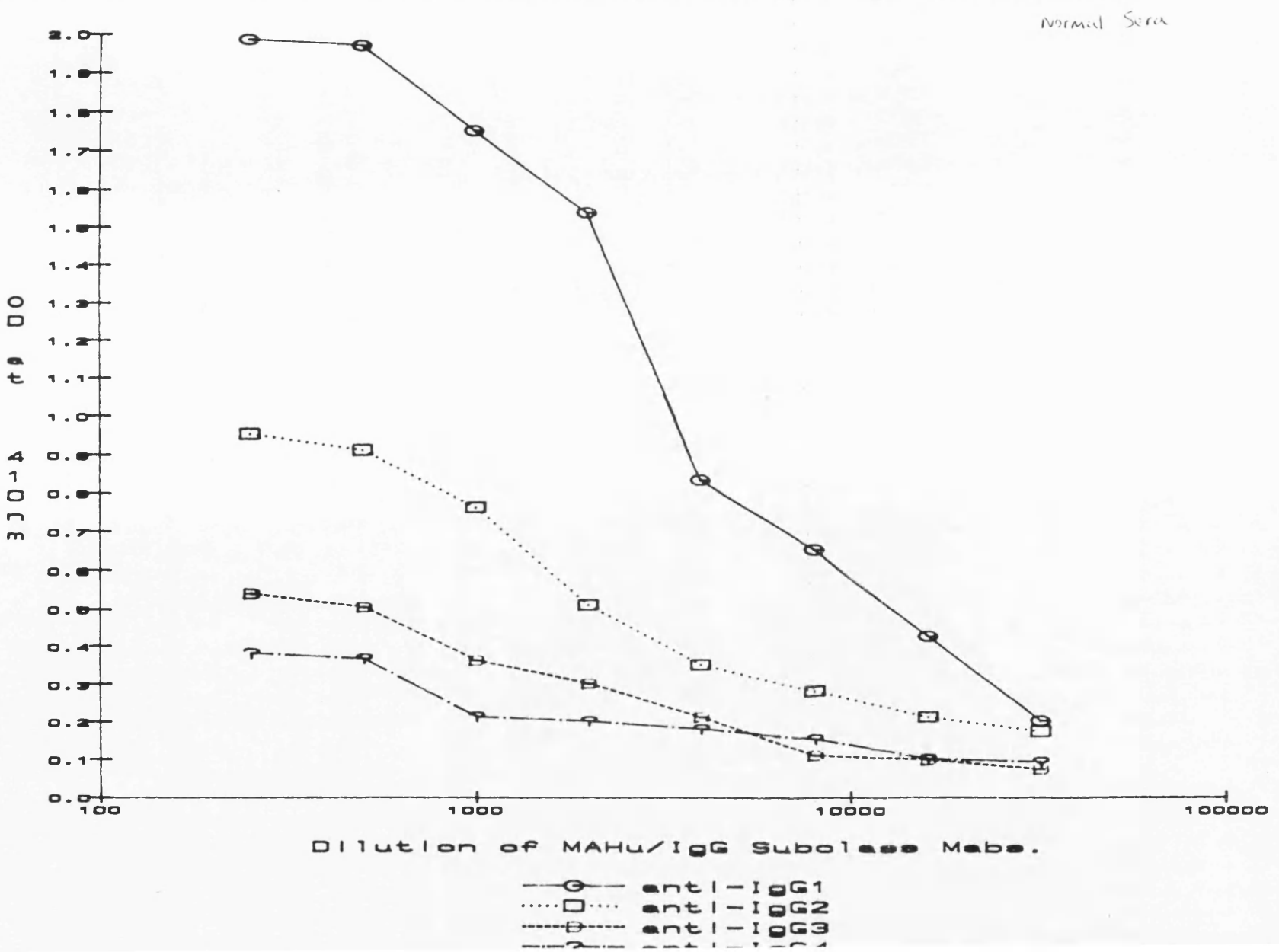
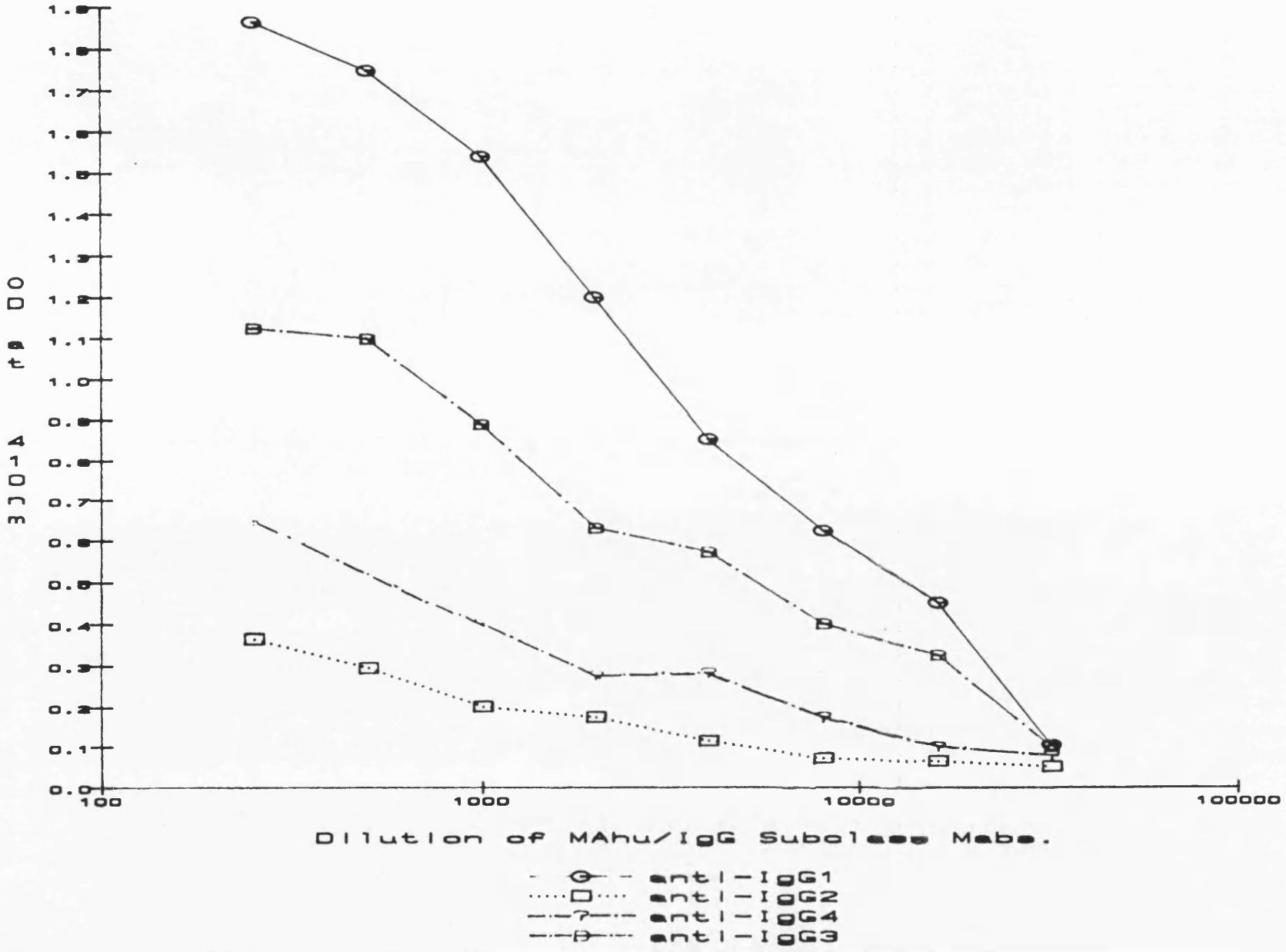

Titration of Mouse anti-human IgG subclasses
Normal Sera
ELISA at OD
2.0
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
100
1000
10000
100000
Dilution of MAHu/IgG Subclass Mabs.
anti-IgG1
anti-IgG2
anti-IgG3

Figure 3.2(b). Titration of Mouse anti-human IgG subclasses - Diabetic Patient



In Figure 3.2.(b), anti-IgG subclasses tirated against insulin coated plate is shown. A microtiter plate was coated with 15 ug.ml^{-1} human insulin, followed by serum from a diabetic patient known to have high insulin antibody titer. Anti-IgG subclasses were titrated across the plate in duplicates, and the distribution of IgG subclasses were detected using GAM/Ig(Y)/PO, as described in section 4.3.2.

Both Figure 3.2.(a) and (b) show optimum dilution for all four anti-IgG subclasses to be 1 in 500.

In Figure 3.3., titration of GAM/Ig(Y)/PO shows that optimum dilution is 1 in 1000.

Figure 3.4. illustrates the titration of MAHu/IgM, anti-M IgG.HRP, and GAHu/IgE (Fc)/PO. A 96 well microtiter plate was coated with 1 in 30 diluted serum sample from a diabetic patient. A two-way titration of MAHu/IgM and anti-M IgG.HRP was made as follows: MAHu/IgM was titrated across the plate at 4 different dilutions (1 in 100; 1 in 200; 1 in 400; 1 in 800). For each dilution, 8 wells were used. To the remaining wells PBS Tween 20 was added. Following 1 hour incubation at room temperature, the plate was washed and anti-M.IgG.HRP was titrated down the plate (in duplicates) in wells coated with MAHu/IgM. In the remaining wells, GAHu/IgE(Fc)/Po was tirated. The results (Figure 3.4.) show that optimum dilutions for MAHu/IgM was 1 in 100, for anti-M.IgG.HRP was 1 in 150, and for GAHu/IgE(Fc)/PO was 1 in 200. The

standard error bars for duplicate wells are not shown because they were very small (<1%).

Figure 3.3. Titration of Goat anti-Mouse.HRP

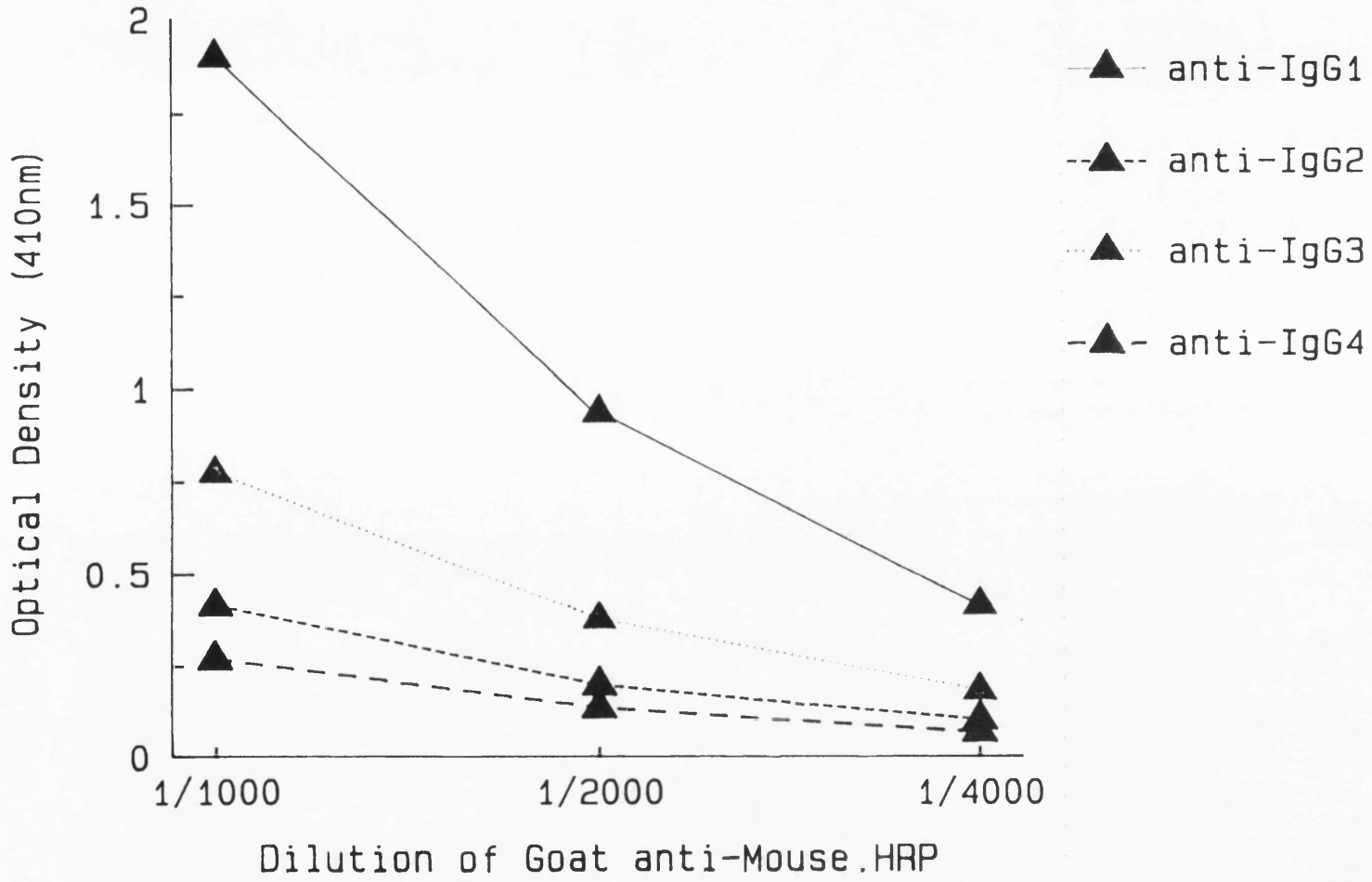
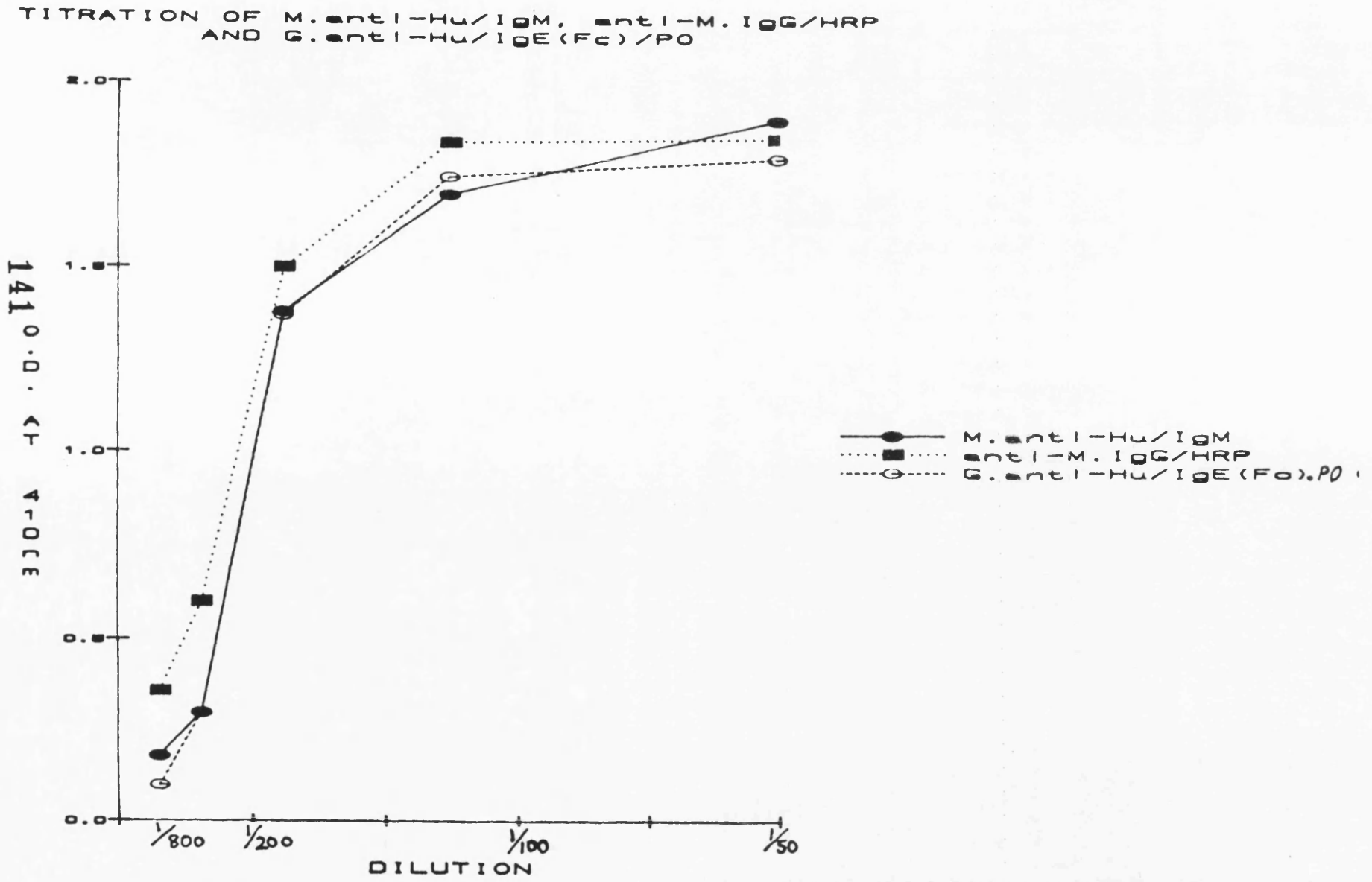


Figure 3.4.


TITRATION OF M.anti-Hu/IgM, anti-M.IgG/HRP
AND G.anti-Hu/IgE(Fc)/PO
2.0
1.5
1.0
0.5
0.0
1410 O.D. 492 nm
1/800
1/200
1/100
1/50
DILUTION
M.anti-Hu/IgM
anti-M.IgG/HRP
G.anti-Hu/IgE(Fc).PO

3.4. *RESULTS*

The humoral immune response to insulin by insulin treated diabetic patients was measured in terms of total anti-insulin IgG in serum. Anti-insulin antibody titer was determined using an Enzyme Linked Immunosorbent Assay (ELISA).

The O.D. readings (at $_{410nm}$) of individual wells of a 96-well microtiter plate were captured on disc and subsequently analysed by a `four parametric logistic fit' model (Rodbord & Hutt, 1974) using a programme on the Eli Lilly corporate computer. This model was used because the four parameters of a curve, namely, zero response, slope, EC_{50} and infinite response (Max) are not fixed in an ELISA. In each microtiter plate serial dilution of a standard with known high anti-insulin antibody content was made, the anti-insulin antibody concentration in the serum of individual patients and controls were determined by extrapolation from the standard curve. Figure 3.5. shows a typical standard curve. The mean + SD of the four parameters of 75 such curves are tabulated below.

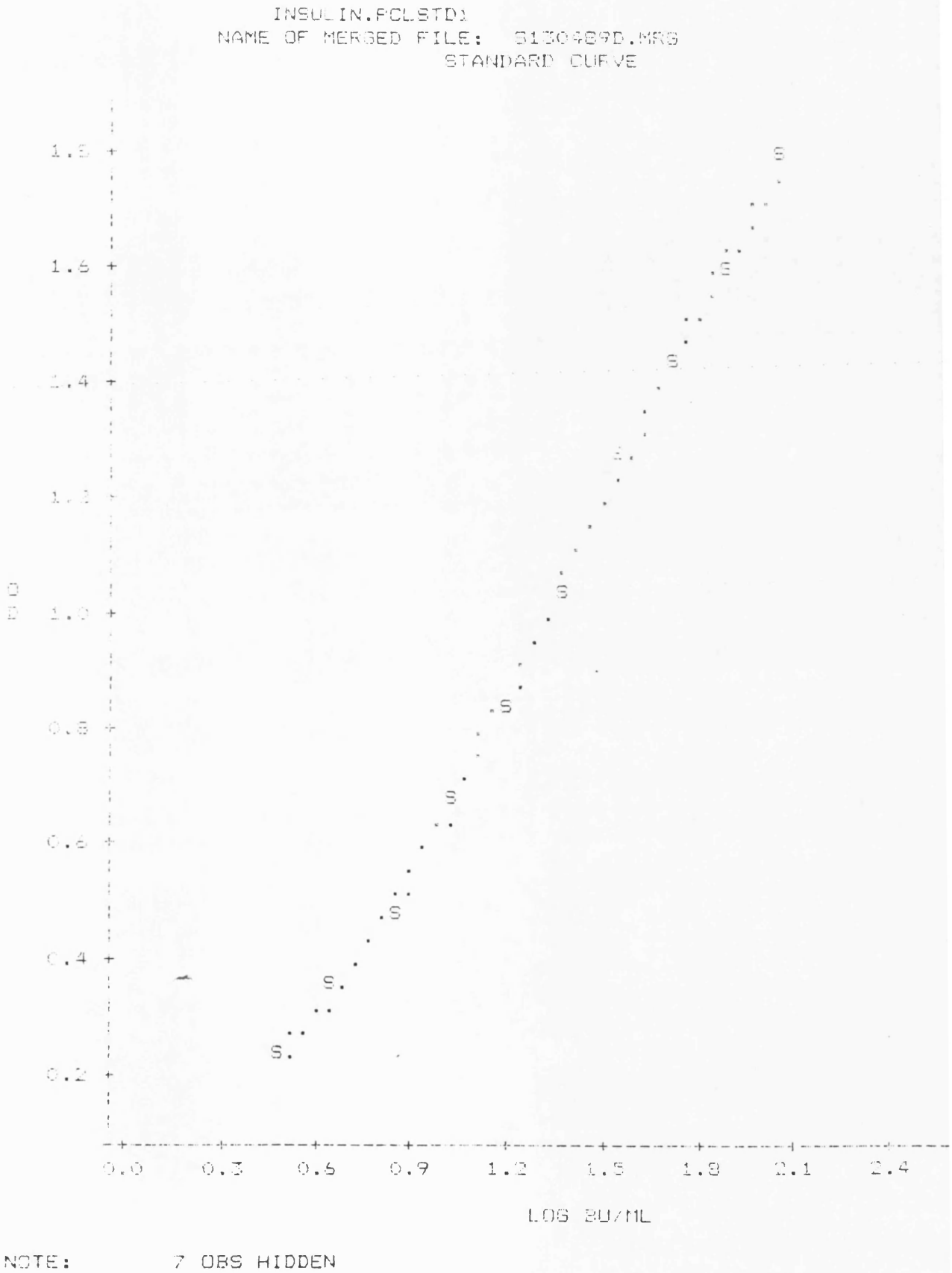
Table 3.2.

Statistical summary of 75 standard curves:

Parameter	N	Mean	SD
Zero Response	75	0.0087	0.082
Slope	75	1.1781	0.302
EC50	75	26.439	11.08
Inf. Response	75	2.2230	0.295

Minimum detectable concentration = 4.5988 binding units*
Maximum measurable concentration = 176 binding units*

Figure 3.5.


INSULIN.PCLSTD1
NAME OF MERGED FILE: S130489D.MRG
STANDARD CURVE
1.8
1.6
1.4
1.1
1.0
0.8
0.6
0.4
0.2
O
D
0.0
0.3
0.6
0.9
1.2
1.5
1.8
2.1
2.4
LOG BU/ML
NOTE: 7 OBS HIDDEN

* The results were determined in terms of `binding units' and later converted to `ug.ml^{-1}, (see` methods' section 4.3.5.) 1 binding unit = 3.45 ug.ml^{-1} of serum.

Each plate also contained negative and high response controls. The coefficient of variation of 60 plates was 6% for the positive control and 8 % for the negative control. Serum samples were analysed in duplicate. In most cases, the standard deviation between the two replicates was less than 3%; samples which gave a SD value greater than 10% were reassayed.

3.4.1. *Anti-Insulin Immunoglobulin G*

Patients On Human Insulin Therapy

Seventy-six IDDM patients were tested for the anti-insulin antibody concentration in their serum. Their clinical details, and total anti-insulin IgG levels are shown in Table 3.3. In Table 3.4. the anti-insulin antibody concentration detected in the serum of non-diabetic control subjects are compared with that of diabetic patients using non-parametric statistics.

Table 3.4. illustrates that the concentration of anti-insulin antibody in the serum of diabetic patients was significantly higher than that of control subjects. The results of control

subjects represents the threshold value of non-specific anti-IgG binding to insulin coated plates (baseline value). A significant antibody response was, therefore, defined as one which exceeded the mean antibody concentration of non-diabetic controls by 2 standard deviation.

Of the 76 Group I patients, 42 (55%) possessed significant anti-human insulin antibody, 39 (51%) had anti-pork insulin antibody and 40 (53%) showed significant anti-beef insulin antibody. In Table 3.5. the difference in concentration of anti-human, anti-pork and anti-beef insulins in the sera of Group I diabetic patients was assessed using a two-sample Wilcoxon test for paired data. The Spearman's correlation coefficient (r_s) between the three types of insulin are also shown in Table 3.5.

Table 3.5. shows that there is a highly significant difference in the concentrations of the three different types of anti-insulin antibodies. There was also a significant correlation between the three types of anti-insulin antibody titers ($r=0.867$ to 0.898 $P<0.0001$) indicating cross-reactivity of antibody with the three types of insulin. This is most apparent in the level of antibody detected on beef insulin coated plates since most of the Group I patients have not been treated with beef insulin and therefore would not be expected to possess specific anti-beef insulin antibodies.

Table 3.3.

Total anti-insulin IgG concentration ($ug.ml^{-1}$) in sera of Group I diabetic patients.

No.	Clinical Background Age	DID	Dose	%HbA1	Concentration of total IgG anti-HI	anti-PI	anti-BI
1	43	12	0.75	ND	25.88	20.94	35.19
2	24	7	1.03	10.5%	24.19	11.08	16.53
3	28	4.5	0.67	ND	42.33	28.77	48.82
4	42	27	0.76	ND	22.01	13.97	23.63
5	26	17	0.78	10.8%	27.60	14.90	25.81
6	53	28	0.77	10.8%	124.89	52.06	111.44
7	39	9	0.90	10.7%	24.12	21.74	34.02
8	35	23	0.50	15.9%	24.70	16.53	24.08
9	23	10	0.63	15.0%	33.29	23.56	36.71
10	36	2	0.39	13.1%	38.64	16.77	25.01
11	35	5	0.55	13.8%	27.22	24.46	21.05
12	27	4	0.27	13.1%	20.01	19.32	29.22
13	22	4	1.04	12.9%	18.29	13.32	16.56
14	50	3	0.52	13.6%	52.65	14.74	21.74
15	25	1.5	0.31	10.3%	35.54	26.22	42.78
16	45	6	0.66	11.4%	24.12	8.83	16.08
17	31	5	0.65	14.7%	41.40	34.85	40.12
18	40	30	0.66	12.6%	21.87	17.39	18.29
19	22	3	1.06	7.7%	112.57	129.00	110.70
20	47	2	0.50	12.7%	71.07	72.93	62.58
21	34	10	0.61	15.3%	26.77	19.39	19.98
22	19	9	1.13	11.7%	27.70	21.39	17.94
23	17	7	1.69	11.2%	30.36	25.88	29.67
24	53	26	0.78	12.9%	45.75	42.09	42.44
25	41	9	0.84	12.9%	18.63	13.73	26.05
26	28	14	1.01	12.1%	26.91	18.11	27.08
27	44	4	0.26	11.8%	28.74	20.84	37.54
28	31	1	0.53	9.4%	36.54	28.74	36.78
29	54	11	0.61	11.0%	34.05	27.53	24.67
30	39	9	0.71	12.3%	117.65	143.14	99.19
31	27	12	1.62	10.4%	42.44	32.64	32.88
32	46	35	0.80	8.4%	124.23	136.93	117.51
33	33	1	0.31	8.1%	50.96	46.33	37.95
34	16	14	1.65	16.0%	66.93	67.21	44.89
35	40	31	0.51	13.7%	15.84	13.56	8.94
36	27	5	0.83	10.9%	12.59	9.45	4.97
37	35	5	1.14	13.9%	11.70	10.14	6.87
38	50	35	0.54	14.0%	29.33	29.08	27.26
39	44	27	0.51	11.8%	41.30	31.53	28.39
40	51	30	1.92	10.7%	32.60	33.29	55.68
41	29	13	0.88	9.4%	64.27	58.96	58.75
42	37	30	0.86	12.3%	22.67	25.43	19.15
43	34	24	0.34	ND	12.90	9.04	9.21
44	31	7	0.66	14.5%	22.49	18.94	19.32
45	47	25	0.54	13.0%	16.42	14.53	10.52

continued.....

Table 3.3. continued:

	Clinical Background				Concentration of total IgG		
No.	Age	DID	Dose	%HbA1	anti-HI	anti-PI	anti-BI
46	29	1.5	0.23	7.4%	16.56	14.35	11.04
47	30	11	0.80	ND	13.52	11.04	6.00
48	51	21	0.87	10.1%	51.06	48.65	74.87
49	31	21	0.98	12.7%	32.19	26.12	21.98
50	47	24	0.66	9.0%	19.67	16.97	34.33
51	50	20	0.75	ND	29.67	24.70	15.63
52	17	4	1.01	ND	23.29	14.90	13.90
53	30	23	0.69	15.5%	26.84	23.08	17.42
54	35	18	0.69	ND	49.34	53.30	31.22
55	48	4	0.43	9.3%	25.12	22.12	14.11
56	34	7	0.68	8.3%	84.35	55.89	56.41
57	42	22	0.79	11.8%	13.08	7.31	7.31
58	35	21	0.60	ND	25.53	22.77	15.53
59	45	12	0.78	ND	17.04	13.77	12.59
60	33	9	0.77	ND	11.97	6.90	7.31
61	42	16	0.64	ND	13.90	10.83	8.87
62	28	10	1.29	ND	21.39	4.52	11.87
63	48	13	0.77	ND	41.75	57.20	54.48
64	36	26	0.73	ND	15.42	6.35	11.35
65	51	22	0.78	ND	59.89	46.40	31.02
66	39	23	0.94	ND	59.38	55.41	30.74
67	50	6	0.58	ND	25.19	19.60	14.97
68	30	6	0.81	ND	18.63	3.45	10.59
69	17	4	0.28	ND	56.99	90.29	49.34
70	37	8	0.44	ND	4.69	7.90	5.21
71	24	1	0.40	ND	5.35	9.07	6.52
72	34	25	0.86	ND	35.98	45.71	37.36
73	40	NK	NK	ND	16.77	23.22	14.84
74	43	NK	NK	ND	8.28	11.94	8.31
75	25	NK	NK	ND	8.63	13.04	6.62
76	21	NK	NK	ND	88.42	93.01	77.73
N	76	72	72		76	76	76
mean	35.8	13.5	0.75		35.42	30.77	30.57
SD	10.1	9.8	0.33		26.53	28.72	25.00
med.	35.0	10.5	0.72		26.88	21.56	24.37

anti-HI=anti-human insulin antibody; anti-PI=anti-pork insulin antibody; anti-BI=anti-beef insulin antibody; DID=duration of disease (years); Dose=units/day/Kg body weight; %HbA1=% glycosylated haemoglobin; ND=not determined; NK=not known. No.=patient number; N=number of observations; SD=standard deviation; med.=median value.

Table 3.4.

Comparison of anti-insulin IgG concentration ($ug.ml^{-1}$) in the sera of Group I patients with non-diabetic controls:

	Type & Concentration	of Anti-Insulin IgG	
	Human	Pork	Beef
NON-DIABETIC CONTROLS N	22	22	22
mean	15.31	12.32	12.62
SD	4.98	4.7	5.32
median	14.92	14.92	12.08
*(mean + 2 SD)	25.27	21.72	23.26
GROUP I DIABETIC PATIENTS: N	76	76	76
mean	35.42	30.77	30.57
SD	26.53	28.72	25
median	26.88	21.56	24.37
Mann-Whitney U =	231	339.5	339
z =	5.01	4.04	4.05
Probability >z =	0	0	0

**non-specific binding = > mean anti-insulin antibody concentration of control sera + 2 SD. N=number of patients. SD=standard deviation. z=standard normal deviate.*

Table 3.5.

Difference (Wilcoxon T) & Correlation (Spearman's r_s) in concentration of anti-human, anti-pork and anti-beef insulin IgG in the sera of Group I patients:

Type of anti-insulin IgG	N	Wilcoxon T	z	P	(r_s)*
Human vs Pork:	76	634	4.29	0.0	0.898
Human vs Beef:	76	630	4.31	0.0	0.889
Pork vs Beef:	75	163	0.169	NS	0.867

** all correlation values were highly significant (P<0.001).*
N=number of patients
z=standard normal deviate
P= probability > z

Group I diabetic patients who gave positive antibody response were divided into three sub-groups on the basis of their anti-insulin antibody concentration.

Low responders: > negative response + 2 SD; < mean value for all patients on human insulin therapy.

Medium responders: > mean; < mean + 2 standard deviation.

High responders: > mean + 2 SD.

On the basis of the above definitions, the number and percentage of Group I patients in each group are tabulated in Table 3.6. Among Group I diabetic patients, eight subjects have been exposed to human insulin therapy only. Of these, 4 gave negative responses, 2 gave low responses and the remaining 2 gave medium responses, the maximum response was 50.96 $ug.ml^{-1}$ serum.

Patients' `age', `duration of diabetes', `duration of insulin therapy', `daily insulin dose requirement' and `diabetic control' were all considered for their potential effect on the antibody titer. In Table 3.7., the influence of these factors on the level of anti-insulin antibody concentration are analysed.

Table 3.6.

Antibody responder status of 76 Group I diabetic patients:

Responder Status		Type of anti-insulin IgG anti-HI	anti-PI	anti-BI
Negative Response	=	<25.3*	<21.7*	<23.3*
	N	34	38	36
	%	44.7%	50.0%	47.4%
Low Response	=	25.3-35.4*	21.7-30.8*	23.3-30.6*
	N	16	16	11
	%	21.1%	21.0%	14.5%
Medium Response	=	35.5-88.5*	30.9-88.2*	30.7-80.6*
	N	22	17	25
	%	28.9%	22.4%	32.9%
High Response	=	>88.5*	>88.2*	>80.6*
	N	4	5	4
	%	5.3%	6.6%	5.3%

Values were rounded-off to one decimal point.
**=anti-insulin IgG concentration in ug.ml^{-1} serum.*
N=number of patients in each group.
%=percentage of patients in each group.

Table 3.7.

Relationship between clinical background and sera anti-insulin IgG concentration. Spearman's Rank Correlation Coefficient (r_s) values:

		Concentration of Anti-Insulin IgG ($ug.ml^{-1}$)		
		anti-HI	anti-PI	anti-BI
AGE (years)	r_s=	0.0690	0.0835	0.0661
	P=	NS	NS	NS
DURATION OF DISEASE (years)	r_s=	0.001	0.0963	0.0272
	P=	NS	NS	NS
DURATION OF INSULIN THERAPY (years)	r_s=	0.0087	0.1023	0.0296
	P=	NS	NS	NS
DAILY INSULIN DOSE (U/D/Kg)	r_s=	0.0779	0.0660	0.0794
	P=	NS	NS	NS
DIABETIC CONTROL (% HbA1)*	r_s=	-0.2330	-0.2475	-0.3010
	P=	NS	NS	< 0.05

*number of patients=72. *=number of patients=49. anti-HI = anti-human insulin antibody; anti-PI = anti-pork insulin antibody; anti-BI = anti-beef insulin antibody. r_s=Spearman rank correlation coefficient. P=probability. NS=not significant.*

Among Group I diabetic patients there were 46 male and 30 female, the antibody response to insulin was found not to be influenced by sex (z=0.319; P=NS).

Further aspects of the relationship between patients' daily insulin dose and the level of anti-insulin antibody were investigated. There was no relationship between dose and antibody titer even when patients with extremely high (>1.08 U/D/Kg (= mean daily insulin dose of all patients + 1 SD)) and low (<0.422 U/D/Kg (=mean daily insulin dose of all patients - 1 SD)) dose were used in the analysis. The duration of disease

of patients with low insulin dose, was however, significantly lower than those on high insulin dose (Mann-Whitney U=5; z=2.56; P=0.0053). The overall correlation between insulin dose and duration of disease was not significant.

Table 3.7. shows an apparent inverse correlation between % HbA1 level and antibody titer. However, the correlation just fails to be significant with anti-human and anti-pork insulin antibodies. Figure 3.6. shows the relationship between % HbA1 value and anti-beef insulin antibody concentration. In order to investigate the relationship between diabetic control and antibody concentration in detail, Group I diabetic patients were divided into three sub-groups on the basis of their glycosylated haemoglobin level: those with a stable glycosylated haemoglobin level of < 11% determined on several occasions over a one year period are thought to have well controlled diabetes; those with > 13% HbA1 and frequent change in insulin dose are thought to have poorly controlled diabetes; the remaining patients with % HbA1 within 10-13% are classified as patients with intermediate control. In Table 3.8., Mann-Whitney U test was used to determine whether the antibody concentration of patients with good diabetic control was significantly higher than in those with poor diabetic control.

Patients with good diabetic control were found to have

significantly higher anti-insulin antibody titer than patients with poor diabetic control. The difference in antibody concentration in sera of patients with intermediate diabetic control did not significantly differ from that of patients with poor or good diabetic control.

Figure 3.6.

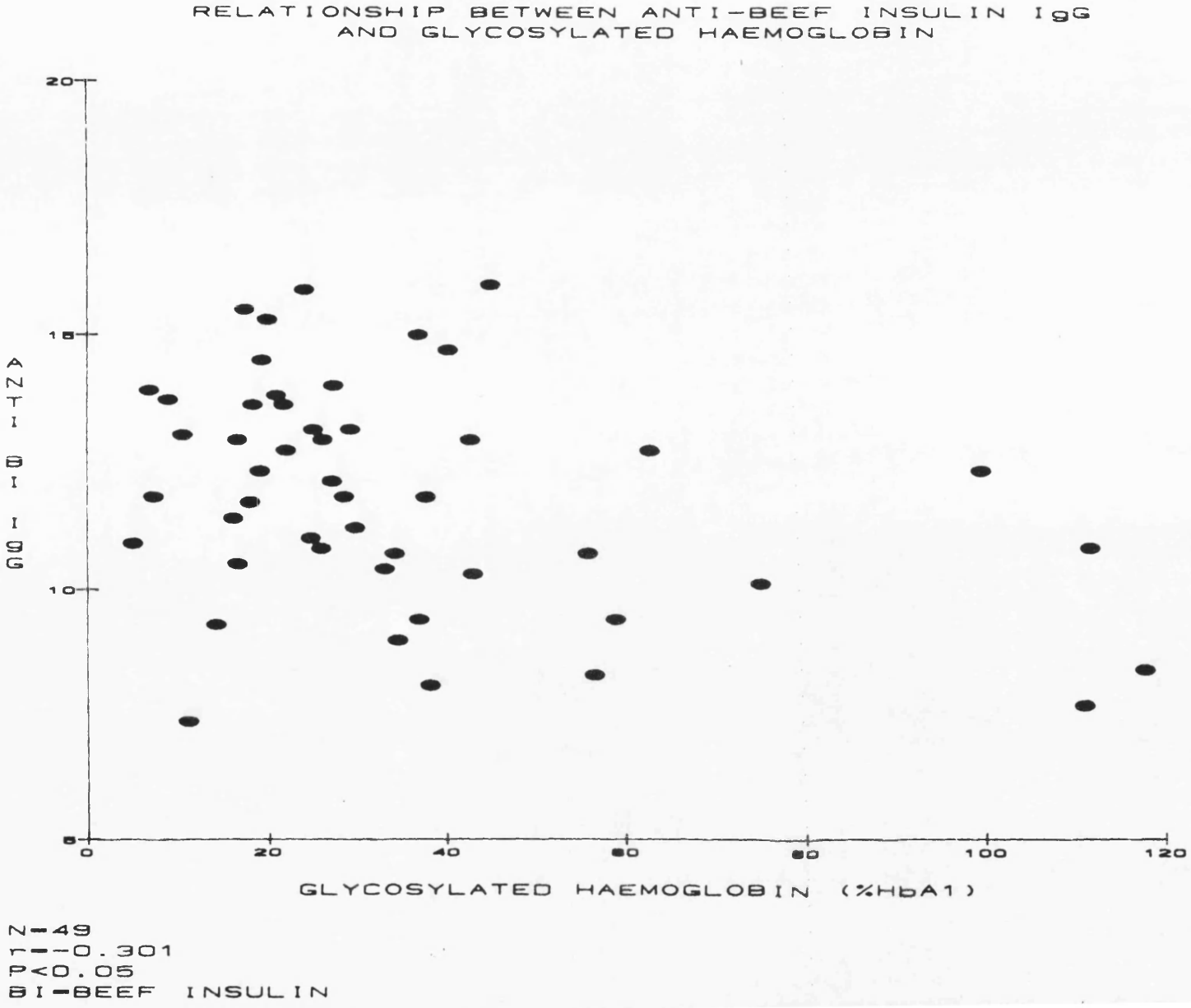

RELATIONSHIP BETWEEN ANTI-BEEF INSULIN IgG
AND GLYCOSYLATED HAEMOGLOBIN
ANTI BI IgG
20
15
10
5
0
20
40
60
80
100
120
GLYCOSYLATED HAEMOGLOBIN (%HbA1)
N=49
r=-0.301
P<0.05
BI=BEEF INSULIN

Table 3.8.

Relationship between diabetic control and anti-insulin IgG concentration.

		Concentration Of Anti-Insulin IgG ($ug.ml^{-1}$)		
		anti-HI	anti-PI	anti-BI
PATIENTS WITH GOOD DIABETIC CONTROL (<11% HbA1)	N	19	19	19
	mean	49.65	41.4	47.44
	SD	10.43	10.35	9.90
	median	35.54	28.74	36.78
PATIENTS WITH INTERMEDIATE CONTROL (HbA1 11-13%)	N	15	15	15
	mean	36.05	32.50	31.33
	SD	7.72	10.02	6.67
	median	27.70	21.39	26.05
PATIENTS WITH POOR DIABETIC CONTROL (HbA1 >13%)	N	15	15	15
	mean	27.98	23.03	23.31
	SD	3.94	3.99	3.09
	median	26.77	19.32	21.75
Mann-Whitney U*		85.0	91.0	74.0
z		1.99	1.79	2.38
Probability**		0.023	0.037	0.0088

*anti-HI=anti-human insulin antibody; PI=anti-pork insulin antibody; BI=anti-beef insulin antibody. z=standard normal deviate. *Mann-Whitney U value for testing the significance of difference between patients with good and poor diabetic control. **P value is given for a one-tailed test; For a two-tailed test the P values should be doubled.*

3.4.2. *Anti-Insulin IgG Subclasses*

Positive sera from Group I diabetic patients were analysed for their anti-insulin IgG subclass distribution. For each patient, the sum of IgG1, 2, 3 and 4 was calculated and taken as 100% value in order to assess the relative abundance of each IgG subclass implicated in anti-insulin immune response. Table 3.9. shows the distribution of anti-human insulin IgG subclasses in the serum of individual diabetic patients as well as the group mean +SD and the patients' clinical background..

The results showed that all patients possessed anti-insulin IgG1 and in most cases, it was the predominant anti-insulin IgG subclass. The next most prevalent subclass was IgG3 followed by IgG2 and IgG4. The distribution of anti-insulin IgG2 and IgG4 varied considerably from patient to patient. For example, in patients 24 and 34 IgG2 was the most prevalent subclass at 58.6% and 38.8% respectively. Patient 30 on the other hand lacked any detectable anti-insulin IgG2. Similarly, in five patients the IgG4 subclass reached as high as 27-34%, yet it was not detected in two patients (nos. 10 and 23). All patients possessed anti-insulin IgG3; in seven patients it was the dominant subclass.

As with total IgG concentration, IgG subclass distribution was thought to be potentially influenced by the clinical background of the patients. These possible relationships are analysed in Table 3.10.

There was a significant inverse correlation between the level of IgG2 (relative to the other IgG subclasses) and patients' insulin dose, indicating that the higher the patients' daily insulin dose requirement the lower was the level of IgG2. Figure 3.7. represents the relationship. Anti-insulin IgG4, on the other hand, increased with a rise in insulin dose ($r=0.416$; $P<0.01$), as is shown in Figure 3.8. There was also a statistically significant inverse correlation between % IgG4 and age ($r=-0.397$; $P<0.01$). However, Figure 3.9. which represents the correlation, does not show a clear-cut relationship. Anti-insulin IgG1 and IgG3 levels did not affect and/or was not affected by any of the clinical parameters investigated.

Table 3.9.

Clinical background and distribution of anti-insulin IgG subclass in sera of Group I diabetic patients. Results are expressed as relative abundance.

Patient No.	Clinical Background AGE	DID	Dose	HbA1	Relative % IgG subclass IgG1	IgG2	IgG3	IgG4
1	43	12	0.75	ND	36.84	11.58	34.42	17.16
2	28	4.5	0.67	ND	45.06	18.60	23.25	13.10
3	26	17	0.78	10.8%	35.71	12.54	30.20	21.56
4	53	28	0.77	10.8%	36.90	11.57	28.53	23.00
5	23	10	0.63	15.0%	52.58	9.23	24.30	13.80
6	36	2	0.39	13.1%	54.17	13.38	21.80	10.66
7	35	5	0.55	13.8%	35.34	32.03	30.22	2.41
8	50	3	0.52	13.6%	36.23	7.62	53.77	2.38
9	25	1.5	0.31	10.3%	38.54	36.67	18.77	6.02
10	31	5	0.65	14.7%	54.83	4.26	40.92	0.00
11	22	3	1.06	7.7%	55.01	5.62	28.68	10.69
12	47	2	0.50	12.7%	80.44	4.39	12.97	2.20
13	34	10	0.61	15.3%	46.36	15.45	27.11	11.08
14	19	9	1.13	11.7%	48.31	4.24	25.34	22.12
15	17	7	1.69	11.2%	48.36	5.74	16.48	29.43
16	53	26	0.78	12.9%	44.27	16.51	30.10	9.13
17	28	14	1.01	12.1%	47.32	10.42	34.37	7.89
18	44	4	0.26	11.8%	28.63	15.35	50.83	5.19
19	31	1	0.53	9.4%	43.43	15.07	34.42	7.09
20	54	11	0.61	11.0%	52.51	19.85	22.82	4.82
21	39	9	0.71	12.3%	22.21	15.15	58.93	3.71
22	27	12	1.62	10.4%	38.41	6.30	22.39	32.90
23	46	35	0.80	8.4%	63.24	2.06	34.71	0.00
24	33	1	0.31	8.1%	20.00	58.61	19.30	2.09
25	16	14	1.65	16.0%	59.96	1.60	4.74	33.70
26	50	35	0.54	14.0%	59.46	6.08	30.24	4.22
27	44	27	0.51	11.8%	49.45	10.62	26.23	13.70
28	51	30	1.92	10.7%	41.09	12.00	28.82	18.09
29	29	13	0.88	9.4%	72.53	16.42	9.74	1.32
30	51	21	0.87	10.1%	80.82	0.00	15.64	3.54
31	31	21	0.98	12.7%	26.89	8.22	34.05	30.84
32	50	20	0.75	ND	36.79	17.62	41.71	3.89
33	30	23	0.69	15.5%	28.62	7.07	58.84	5.47
34	35	18	0.69	ND	25.19	38.83	27.44	8.55
35	34	7	0.68	8.3%	37.50	33.49	25.00	4.01
36	35	21	0.60	ND	52.80	5.94	36.71	4.55
37	48	13	0.77	ND	74.49	5.67	17.21	2.63
38	51	22	0.78	ND	42.90	12.87	40.76	3.47
39	39	23	0.94	ND	69.64	3.64	24.70	2.02
40	17	4	0.28	ND	69.00	6.24	18.83	5.96
41	34	25	0.86	ND	60.12	1.15	16.79	21.95
42	30	15	1.15	ND	48.57	3.94	20.60	26.90
mean	36	14	0.79	11.79%	47.63	12.94	28.63	10.79
SD	11	9.7	0.37	2.28%	15.29	11.75	12.09	9.77
med.	34.5	12.5	0.73	11.8%	46.84	10.52	27.28	6.55

AGE (years); DID=duration of diabetes (years); Dose (Units/day/Kg body weight); HbA1=%Glycosylated Haemoglobin. ND=not determined; med.=median value; SD=standard deviation.

Table 3.10.

Relationship between clinical background and serum anti-insulin IgG subclass distribution. Spearman's Rank Correlation Coefficient values (r_s):

Anti-Insulin IgG Subclasses	IgG1	IgG2	IgG3	IgG4
AGE (yrs)	-0.0303 NS	0.1425 NS	0.2964 NS	-0.3970 P<0.01
DURATION OF DISEASE (yrs)	0.1134 NS	-0.2891 NS	0.1737 NS	0.1425 NS
DOSE (U/D/Kg)	0.1423 NS	-0.4399 P<0.01	-0.1482 NS	0.4164 P<0.01
% HbA1	-0.0002 NS	-0.1900 NS	0.2281 NS	0.1285 NS

Number of patients = 42.
NS=not significant.
P=probability.

Figure 3.7.

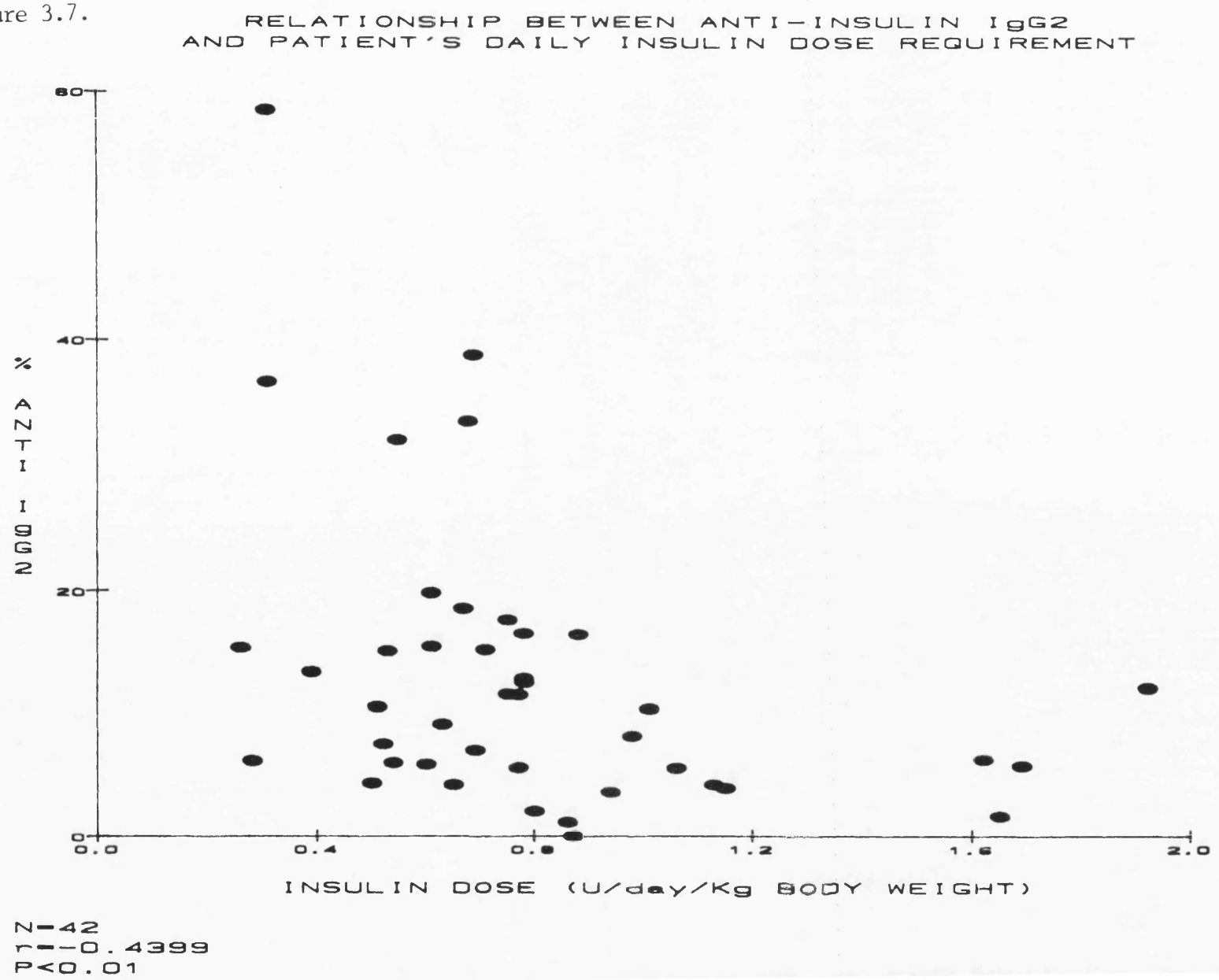

RELATIONSHIP BETWEEN ANTI-INSULIN IgG2
AND PATIENT'S DAILY INSULIN DOSE REQUIREMENT
% ANTI IGG2
60
40
20
0
0.0
0.4
0.8
1.2
1.6
2.0
INSULIN DOSE (U/day/Kg BODY WEIGHT)
N=42
r=-0.4399
P<0.01

Figure 3.8.

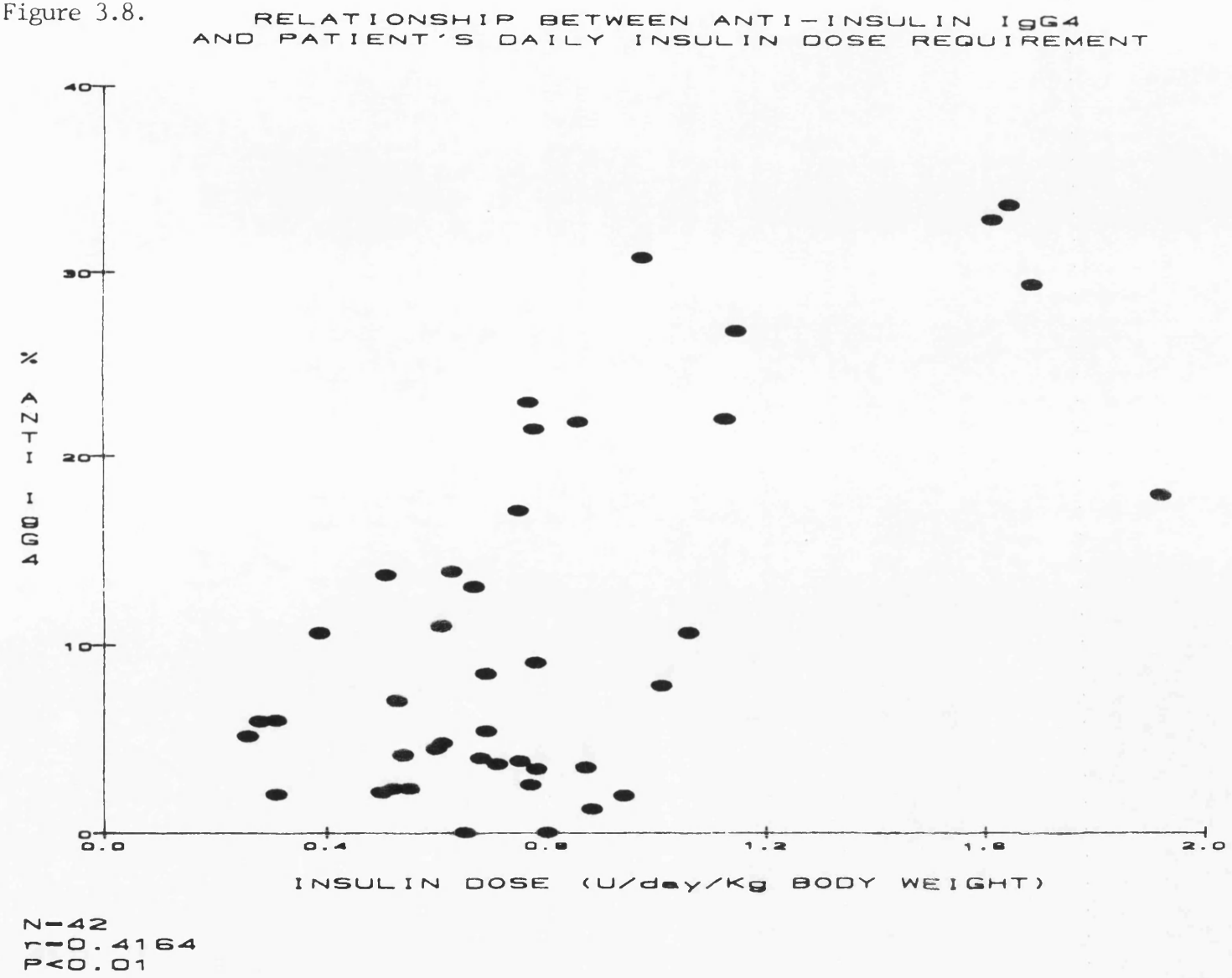
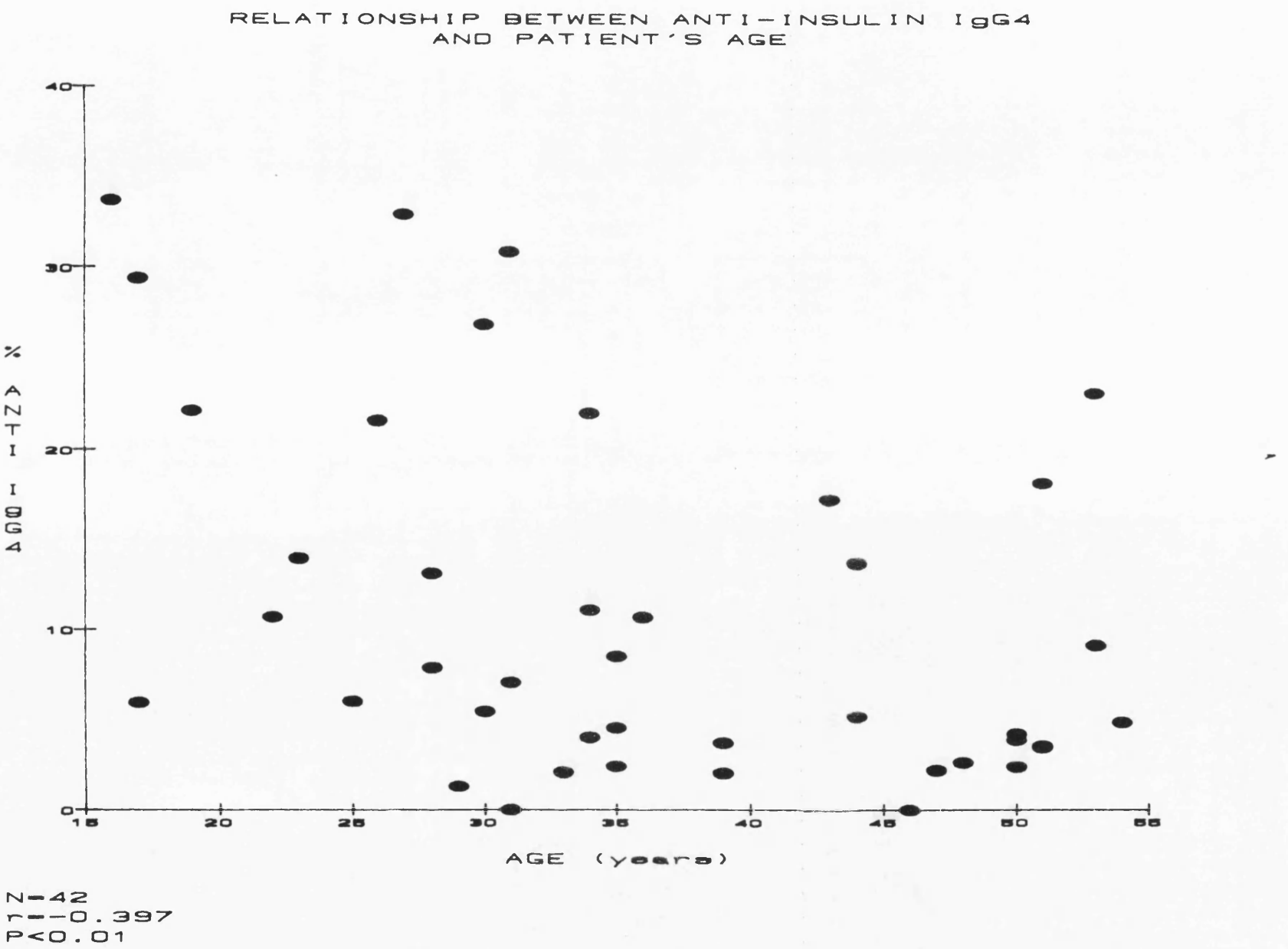

RELATIONSHIP BETWEEN ANTI-INSULIN IgG4
AND PATIENT'S DAILY INSULIN DOSE REQUIREMENT
ANTI-INSULIN IgG4 (%)
0
10
20
30
40
0.0
0.4
0.8
1.2
1.6
2.0
INSULIN DOSE (U/day/Kg BODY WEIGHT)
N=42
r=0.4164
P<0.01

Figure 3.9.


RELATIONSHIP BETWEEN ANTI-INSULIN IgG4
AND PATIENT'S AGE
% ANTI IGG4
40
30
20
10
0
15
20
25
30
35
40
45
50
55
AGE (years)
N=42
r=-0.397
P<0.01

3.4.3. *Patients Transferred From Beef to Human Insulin Therapy (Group II).*

The level of anti-insulin antibody detected in the sera of control subjects represented non-specific binding of antibody to insulin coated plates. Therefore, in order to determine whether significant levels of anti-insulin antibodies are present in the sera of Group II diabetic patients, Mann-Whitney U test was used to compare the serum anti-insulin IgG concentration of Group II patients with control subjects. Table 3.11., which tabulates the results, shows that all three serum samples from Group II diabetic patients contained significantly higher anti-insulin antibody compared to control samples.

Table 3.12. shows the difference in concentration of the three types of anti-insulin antibodies as well as their correlation value. The difference in concentrations of the three types of anti-insulin antibody was analysed using a two-sample Wilcoxon T-test for paired data and the correlation was determined using Spearman's Rank Correlation Coefficient test (r_s). Although there were highly significant correlations in the concentration of anti-human, anti-pork and anti-beef insulin IgG antibodies, the concentration of anti-beef insulin and anti-human insulin IgG did significantly differ from each other in all three samples. In sample 1 anti-beef insulin concentration also differed significantly from anti-pork insulin antibody concentration.

The possible effect of change in insulin therapy on the humoral immune response to insulin was investigated. All three serum samples from each patient were analysed for total anti-insulin IgG antibody on the same microtiter plate by ELISA. Figures 3.10(a) and 3.10(b) shows the change in anti-beef and anti-human insulin antibody level of individual patient. In Table 3.13. the difference in antibody concentration of the three samples and their statistical significance are tabulated.

Table 3.11.

Difference in anti-insulin IgG concentration of Group II diabetic patients and control subjects:

		Type Of Anti-Insulin IgG		
		Human	Pork	Beef
NON-DIABETIC CONTROLS	N	22	22	22
	mean	15.31	12.32	12.62
	SD	4.98	4.7	5.32
	median	14.92	14.92	12.08
GROUP II DIABETIC PATIENTS: **1st SAMPLE**	N	31	31	31
	mean	47.08	45.30	41.80
	SD	62.55	48.67	92.27
	median	24.25	27.6	18.49
	Mann-Whitney U	111	56.5	160
	z	4.15	5.14	3.27
	P	0.0	0.0	0.001
2nd SAMPLE	N	23	23	23
	mean	35.15	31.12	27.63
	SD	25.58	23.14	17.86
	median	27.43	21.67	21.94
	Mann-Whitney U	90	69	82
	z	3.7	4.18	3.88
	P	0.0	0.0	0.0002
3nd SAMPLE	N	24	24	24
	mean	35.70	40.27	42.67
	SD	31.71	35.35	37.52
	median	25.39	31.50	33.81
	Mann-Whitney U	150	124	39
	z	2.51	3.08	4.95
	P	0.0122	0.002	0.0

1st sample - taken prior to change from human to beef insulin therapy; 2nd sample - 3-8 months on human insulin therapy; 3rd sample - >12 on human insulin therapy. z=standard normal deviate. P=probability, significance level.

Table 3.12.

Anti-human, pork & beef insulin antibody: Specificity of antibody and correlation (r_s) in response.

Type of anti-insulin IgG	N	Wilcoxon T	z	P	(r_s)*
1st SAMPLE					
Human vs Pork:	31	218	0.58	NS	0.980
Human vs Beef:	31	118	2.55	0.0108	0.805
Pork vs Beef:	31	57	3.73	0.0002	0.831
2nd SAMPLE					
Human vs Pork:	23	88.5	1.49	NS	0.882
Human vs Beef:	23	29	3.30	0.001	0.935
Pork vs Beef:	23	78	1.81	NS	0.944
3rd SAMPLE					
Human vs Pork:	24	103	1.33	NS	0.933
Human vs Beef:	24	74	2.16	0.031	0.908
Pork vs Beef:	24	123	0.757	NS	0.795

*1st sample - taken prior to change from human to beef insulin therapy; 2nd sample - 3-8 months on human insulin therapy; 3rd sample - >12 on human insulin therapy. N=number of patients. z=standard normal deviate. P= probability > z. * all correlation values were highly significant (P<0.001).*

Table 3.13.

Difference in anti-insulin antibody concentration of 1st, 2nd and 3rd samples.

	Type Of Anti-Insulin IgG		
	Human	Pork	Beef
Difference between 1st and 2nd samples:			
Mann-Whitney U	347	293	323
z	0.166	1.11	0.586
P	NS	NS	NS
Difference between 1st and 3rd samples:			
Mann-Whitney U	334.5	346.5	242
z	0.636	0.433	2.21
P	NS	NS	0.0274
Difference between 2nd and 3rd samples:			
Mann-Whitney U	250	256	188
z	0.553	0.426	1.87
P	NS	NS	NS*

*1st sample - taken prior to change from human to beef insulin therapy; 2nd sample - 3-8 months on human insulin therapy; 3rd sample - >12 on human insulin therapy. z=standard normal deviate. P =significance level; *P=0.061*

Table 3.13. shows that the change in insulin therapy, ie. from beef to human insulin has no significant effect on the anti-insulin IgG concentration. There is, however a significant increase in anti-beef insulin IgG concentration between the 1st and 3rd samples. A closer look at the three samples of individual patients (Figure 3.10.) shows that Group II diabetic patients fall into three basic subgroups with respect to their antibody response to insulin:

Negative responder ie. antibody concentration less than mean + 2 SD of control sera (non-specific binding),

Low to medium responders > non-specific binding + 2 SD; < mean of all three samples + 1 SD (ie. >25.27; <80 $ug.ml^{-1}$).

High responders > mean of all three samples + 1 SD, (ie. >80 $ug.ml^{-1}$).

The low to medium responder did not significantly differ in their antibody response when transferred to human insulin. The high responders did however show a substantial decrease in both anti-human and anti-beef insulin antibody concentration when transferred to human insulin, this is illustrated in Figures 3.10.(a) and 3.10.(b). Unfortunately, the number of patients with high antibody response (numbers 1, 8, 11 and 12 were too few for statistical analysis.

Figure 3.10(a).

Effects of transferring from Bovine to Human Insulin therapy

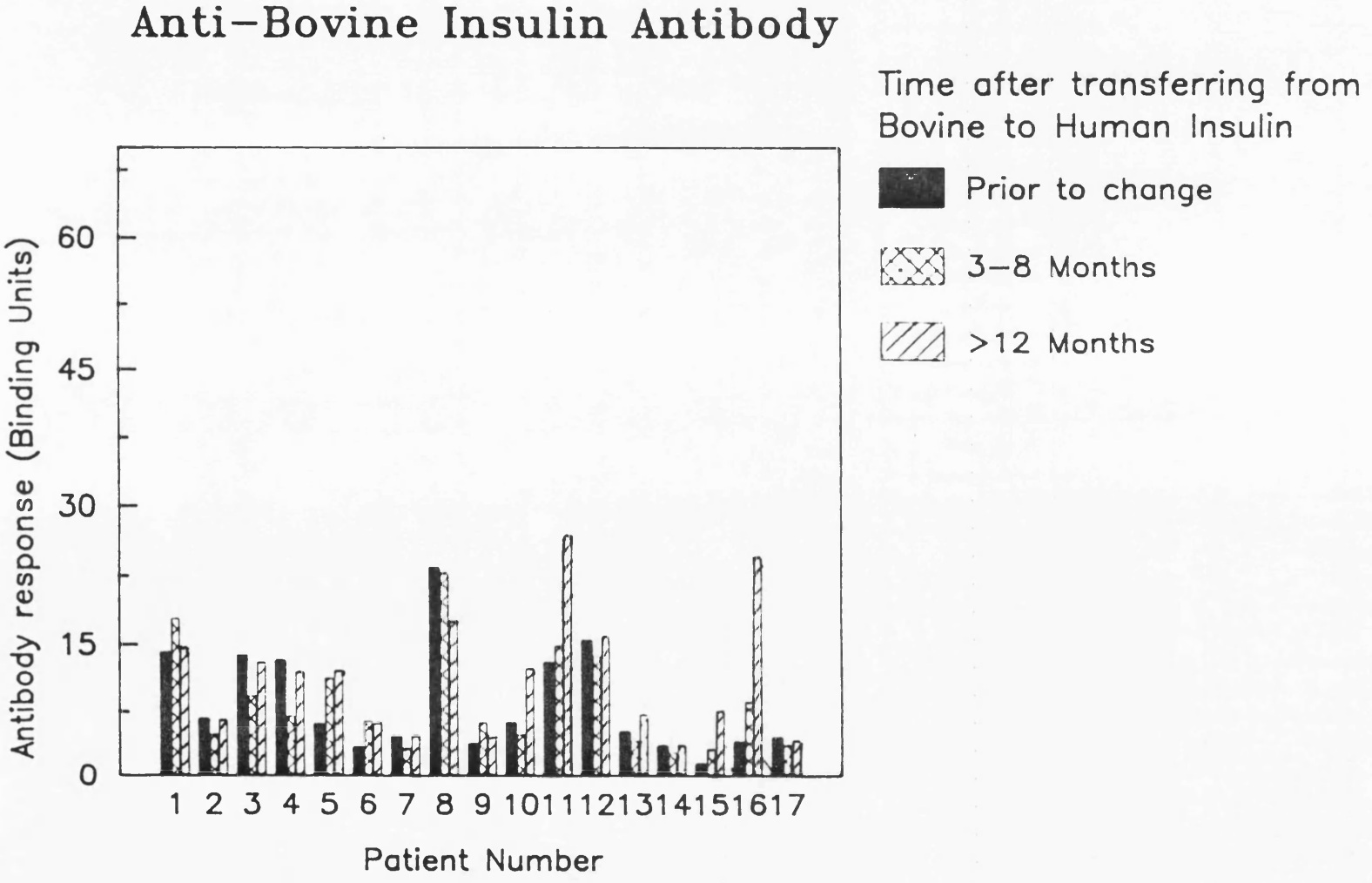
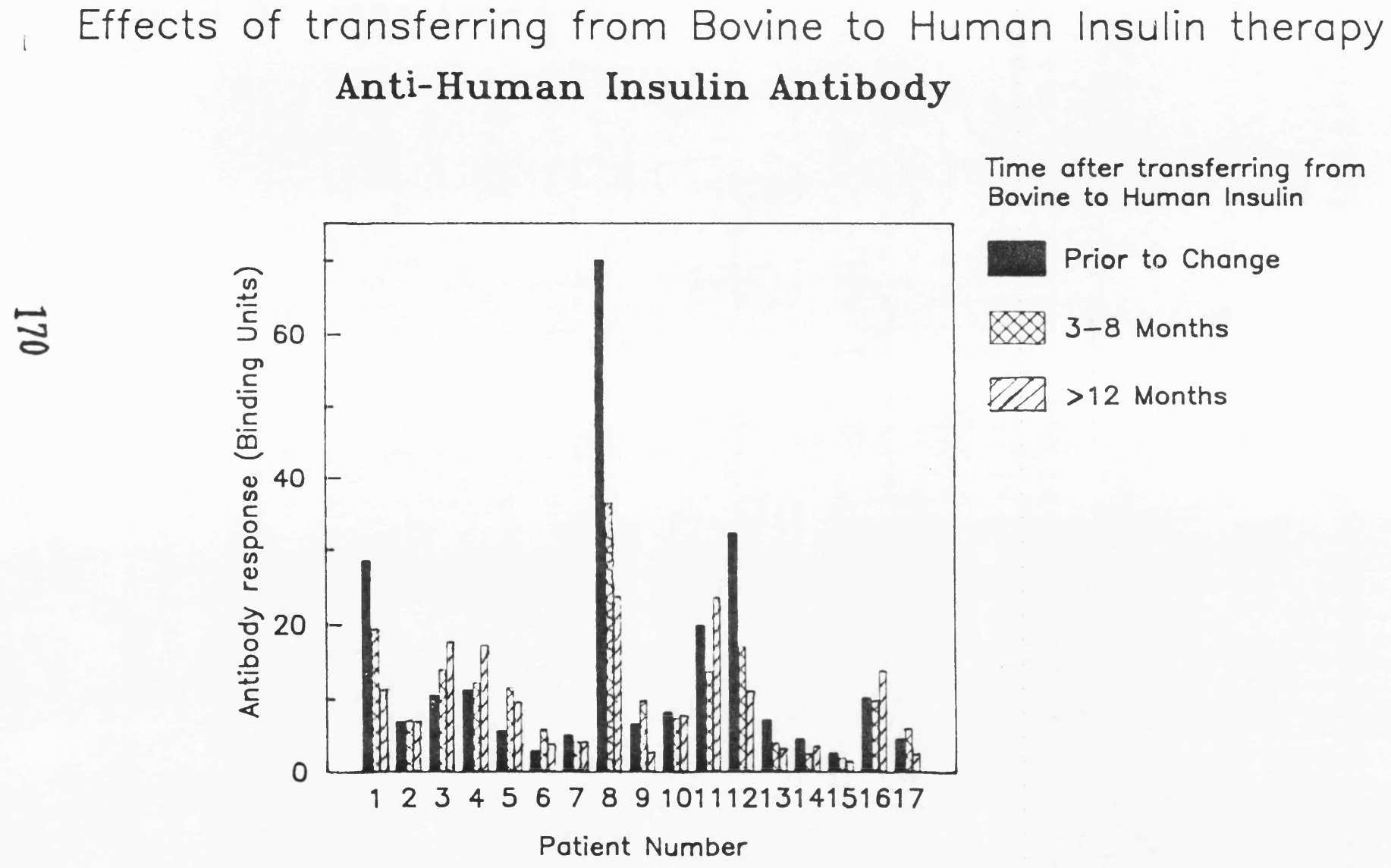


Figure 3.10(b).


Effects of transferring from Bovine to Human Insulin therapy
Anti-Human Insulin Antibody
Time after transferring from Bovine to Human Insulin
Prior to Change
3–8 Months
>12 Months
Antibody response (Binding Units)
0
20
40
60
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Patient Number

3.4.4. *Effects Of Transferring From Beef To Human Insulin Therapy On The Distribution Of Anti-Insulin IgG Subclasses*

Of the 31 Group II diabetic patients, 9 (29%) gave a positive antibody response to insulin while they were on beef insulin therapy (1st sample). The anti-insulin IgG subclass distribution of these positive sera was determined. The results in Table 3.14. indicate that the distribution of anti-insulin IgG subclass of Group II diabetic patients were similar to that of Group I patients. In the sera of patient number 19, the predominant anti-insulin IgG subclass was IgG3 in all three samples. Furthermore, the % anti-human IgG3 level increased from 46.9% (44.9% anti-beef IgG3) to 55.5% (49.6% anti-beef IgG3). In patient number 30, anti-human insulin IgG3 of the 1st sample was not the most abundant subclass (24.6%), but when this patient was transferred to human insulin therapy, anti-human IgG3 increased to 49.6% which exceeded IgG1 (19.4%). The anti-beef insulin IgG4, in this patient also increased from 32.5% to 50.2%. There was also a gradual increase in IgG4 from 45.1% (45% anti-beef) to 51.4% (47.7% anti-beef) in patient number 24. In patient number 22, the anti-human IgG4 increased from 26.1% to 45.1% (see Table 3.14.). In all other patients IgG1 was the predominant subclass.

The distribution of anti-beef insulin IgG subclasses did not differ significantly from the distribution of anti-human insulin IgG subclasses. In fact, there was a highly significant correlation between the two types of anti-insulin IgG subclasses ($r > 0.9$; $P < 0.001$). The distribution of IgG subclasses in the 1st, 2nd and 3rd serum samples were compared. Table 3.15. gives the effect of beef and human insulin therapy on the relative abundance of anti-human and anti-beef insulin IgG subclasses.

Table 3.15. shows that the anti-insulin IgG subclass distribution of 1st, 2nd and 3rd samples did not significantly increase or decrease. One exception being anti-beef IgG2 which decreased from a mean value of 12.8% in the 1st sample to 8.2% in the 3rd sample ($P=0.035$).

Table 3.14.

Anti-human **(anti-beef)** insulin IgG subclass distribution of positive sera from Group II diabetic patients: Results are expressed as relative abundance.

Patient No.	Relative % IgG subclasses IgG1	IgG2	IgG3	IgG4
1st SAMPLE				
4	51.1 (50.9)	11.3 (9.8)	20.4 (21.9)	17.2 (17.4)
5	55.8 (58.0)	7.6 (8.8)	27.5 (23.9)	9.1 (9.3)
8	46.1 (49.4)	7.9 (13.1)	27.1 (27.5)	18.9 (10.1)
14	53.2 (34.7)	14.4 (29.4)	26.5 (16.8)	5.9 (19.1)
19	33.7 (32.8)	8.3 (9.6)	46.9 (44.9)	11.2 (12.7)
21	53.6 (53.2)	12.8 (13.0)	24.5 (25.2)	9.1 (8.6)
22	44.1 (42.7)	14.6 (13.8)	15.2 (18.8)	26.1 (24.7)
24	41.0 (39.3)	4.5 (6.4)	9.5 (9.3)	45.1 (45.0)
30	30.9 (38.7)	12.1 (11.4)	24.6 (17.4)	32.4 (32.5)
mean	45.5 (44.4)	10.4 (12.8)	24.6 (22.8)	19.4 (19.9)
SD	8.9 (8.8)	3.5 (6.7)	10.3 (9.9)	12.9 (12.3)
median	46.1 (42.7)	11.3 (11.4)	24.6 (21.9)	17.2 (17.4)
2nd SAMPLE				
4	57.4 (54.8)	6.0 (5.5)	20.3 (23.0)	16.2 (16.7)
5	67.7 (66.9)	8.9 (10.4)	17.4 (17.1)	6.1 (5.7)
8	45.4 (42.7)	6.3 (5.7)	39.9 (42.3)	8.4 (9.5)
14	57.1 (57.8)	12.3 (11.2)	25.6 (25.8)	4.9 (5.1)
19	29.3 (30.4)	9.6 (7.5)	48.1 (47.3)	12.9 (14.8)
21	62.6 (61.0)	12.3 (12.0)	20.3 (21.9)	4.9 (5.1)
22	48.8 (46.3)	8.9 (9.3)	11.8 (10.8)	30.6 (33.6)
24	41.2 (40.6)	4.6 (6.0)	10.2 (11.2)	43.9 (42.2)
30	ND	ND	ND	ND
mean	51.2 (50.1)	8.6 (8.4)	24.2 (24.9)	16.0 (16.6)
SD	12.5 (12.1)	2.8 (2.6)	13.3 (13.4)	14.2 (14.0)
median	53.0 (50.6)	8.9 (8.4)	20.3 (22.5)	10.7 (12.1)
3rd SAMPLE				
4	54.1 (45.4)	7.1 (5.3)	24.7 (35.9)	14.1 (13.4)
5	73.8 (61.3)	4.4 (6.8)	19.0 (25.4)	2.8 (6.5)
8	ND	ND	ND	ND
14	72.6 (61.5)	8.1 (10.4)	18.2 (22.7)	1.1 (5.4)
19	30.2 (30.9)	5.6 (8.0)	55.5 (49.6)	8.7 (11.5)
21	43.2 (57.3)	13.1 (9.3)	32.0 (23.9)	11.8 (9.4)
22	8.9 (49.1)	14.8 (8.9)	31.2 (16.0)	45.1 (26.1)
24	44.2 (38.3)	0.8 (6.7)	3.7 (7.2)	51.4 (47.7)
30	19.4 (16.8)	10.6 (10.1)	49.6 (22.9)	20.4 (50.2)
mean	43.3 (45.1)	8.1 (8.2)	29.2 (25.5)	19.4 (21.3)
SD	23.4 (15.8)	4.6 (1.8)	17.0 (12.7)	18.9 (18.2)
median	43.7 (47.3)	7.6 (8.5)	28.0 (23.4)	13.0 (12.5)

The relative anti-beef insulin IgG subclasses are shown in brackets. *SD=standard deviation. 1st sample - taken prior to change from human to beef insulin therapy; 2nd sample - 3-8 months on human insulin therapy; 3rd sample - >12 on human insulin therapy.*

Table 3.15.

Statistical summary of the effect of transferring from beef to human insulin therapy on the distribution of anti-human **(anti-beef)** insulin IgG subclasses.

	Anti-Human (Anti-Beef) IgG Subclass			
	IgG1	IgG2	IgG3	IgG4
Difference between 1st and 2nd samples:				
Wilcoxon T	3 (2)	8 (3)	6 (13)	11 (8)
z	1.77(1.94*)	0.93(1.77)	1.27(0.09)	0.42(0.93)
P	NS	NS	NS	NS
Difference between 1st and 3rd samples:				
Wilcoxon T	14 (8)	3 (1)	11 (8)	13.5 (8)
z	0.09(0.93)	1.77(2.11)	0.42(0.93)	0.0(0.93)
P	NS	NS (0.035)	NS	NS
Difference between 2nd and 3rd samples:				
Wilcoxon T	12 (9)	10 (7)	7.5 (7)	10 (14)
z	0.25(0.76)	0.59(1.1)	1.01(1.1)	0.59(0.1)
P	NS	NS	NS	NS

Values for anti-beef insulin IgG subclasses are shown in brackets. *1st sample - taken prior to change from human to beef insulin therapy; 2nd sample - 3-8 months on human insulin therapy; 3rd sample - >12 on human insulin therapy. *P=0.052). N=7. z=normal standard deviate. P=probability.*

3.5. *DISTRIBUTION OF IgM AND IgE IN SERUM*

3.5.1. Group I Patients.

In a preliminary study, the distribution of IgM and IgE in the sera of type I diabetic patients was determined in order the investigate other types of antibody produced in response to insulin therapy and their possible role. Table 3.16. shows the mean + SD of the levels of IgM and IgE protein in the sera of Group I diabetic patients and control subjects. Differences in IgE and IgM of Group I patients were compared with control subjects.

Table 3.16. shows that the distribution of IgM in the sera of Group I diabetic patients was significantly lower than that of control subjects. The distribution of IgE, on the other hand was not significantly different from control subjects. It is widely speculated that IgE is the main type of antibody produced in an allergic reaction. Since insulin allergy has been reported in some patients, it is of interest to investigate the possible relationship between IgE and insulin therapy. Table 3.17. show the relationship between IgE and IgM and the patients' clinical background.

Table 3.17. shows a negative correlation between 'age' and IgE level, i.e. there is a decrease in IgE level with age. This is illustrated in Figure 3.11. In Figure 3.12., the association between patients' daily insulin

dose and IgE is shown. It shows that an increase in insulin dose requirement is correlated with a rise in IgE level. No correlation between IgM and patients' clinical background was established.

3.5.2. *Distribution Of IgM And IgE In The Sera Of Group II Diabetic Patients*

The influence of beef and human insulin therapy on the distribution of IgM and IgE was investigated. Table 3.18. shows the effect of transferring from beef to human insulin therapy on the production of IgE.

The IgE production of Group II diabetic patients did not differ significantly from that of non-diabetic patients. The IgE distribution of 1st, 2nd and 3rd serum samples of Group II diabetic patients did however significantly differ from each other; the IgE titer decreased when the patients were transferred from beef to human insulin therapy. Figures 3.13(a) and 3.13(b) shows the effect of change in insulin therapy on the IgE titer in 16 diabetic patients. Group II diabetic patients also showed a negative correlation between `age' and IgE in their sera, ($N=31$; $r_s=-0.4028$; $P<0.05$). This is illustrated in Figure 3.14.

Table 3.16.

Statistical summary of IgM and IgE in sera of Group I diabetic patients and control subjects: Results are expressed as mean + SD Optical Density (O.D.) at 410nm.

		Type Of Antibody IgM	IgE
NON-DIABETIC CONTROLS	N	22	22
	mean	0.908	0.832
	SD	0.138	0.183
	median	0.941	0.755
GROUP I DIABETIC PATIENTS	N	76	76
	mean	0.741	0.878
	SD	0.103	0.080
	median	0.724	0.860
Difference between Group I patients and control subjects:			
	Mann-Whitney U	291.0	586.5
	z	4.66	1.84
	Probability	0.0	0.066(NS)

N=number of subjects.
z=standard normal deviate.

Table 3.17.

Relationship between IgM & IgE and clinical background (of Group I patients): Spearman's Rank Correlation Coefficient (r_s):

		Patients' Clinical Background AGE	DID	DIT	Dose	% HbA1
Type Of Antibody (O.D. 410nm)						
IgM	N	72	72	72	72	72
	r_s=	-0.0277	0.2263	0.2106	-0.0378	0.052
	P=	NS	NS	NS	NS	
IgE	N	72	72	72	72	72
	r_s=	-0.3064	-0.0334	-0.0296	0.3577	-0.0852
	P=	<0.01	NS	NS	<0.01	NS

AGE (yrs); DID=duration of disease (yrs); DIT=duration of insulin therapy (yrs); Dose (U/day/Kg body weight); % HbA1= glycosylated haemoglobin. N=number of patients. r_s=Spearman rank correlation coefficient. P=probability. NS=not significant.

Table 3.18.

Distribution of IgE in the sera of Group II diabetic patients. Results are expressed as mean +SD O.D. at 410nm:

	Sample Number 1st	2nd	3rd
N	31	24	24
mean	0.787	0.760	0.725
SD	0.057	0.066	0.098
median	0.770	0.740	0.720

Difference between 1st, 2nd & 3rd samples:

	Mann-Whitney U	z	Probability
1st & 2nd Samples:	251.5	2.05	0.0408
1st & 3rd Samples:	210.5	2.74	0.0061
2nd & 3rd Samples:	232.0	1.15	NS

1st sample - taken prior to change from human to beef insulin therapy; 2nd sample - 3-8 months on human insulin therapy; 3rd sample - >12 on human insulin therapy. N=number of patients. SD=standard deviation. z=standard normal deviate. NS=not significant.

Figure 3.11.

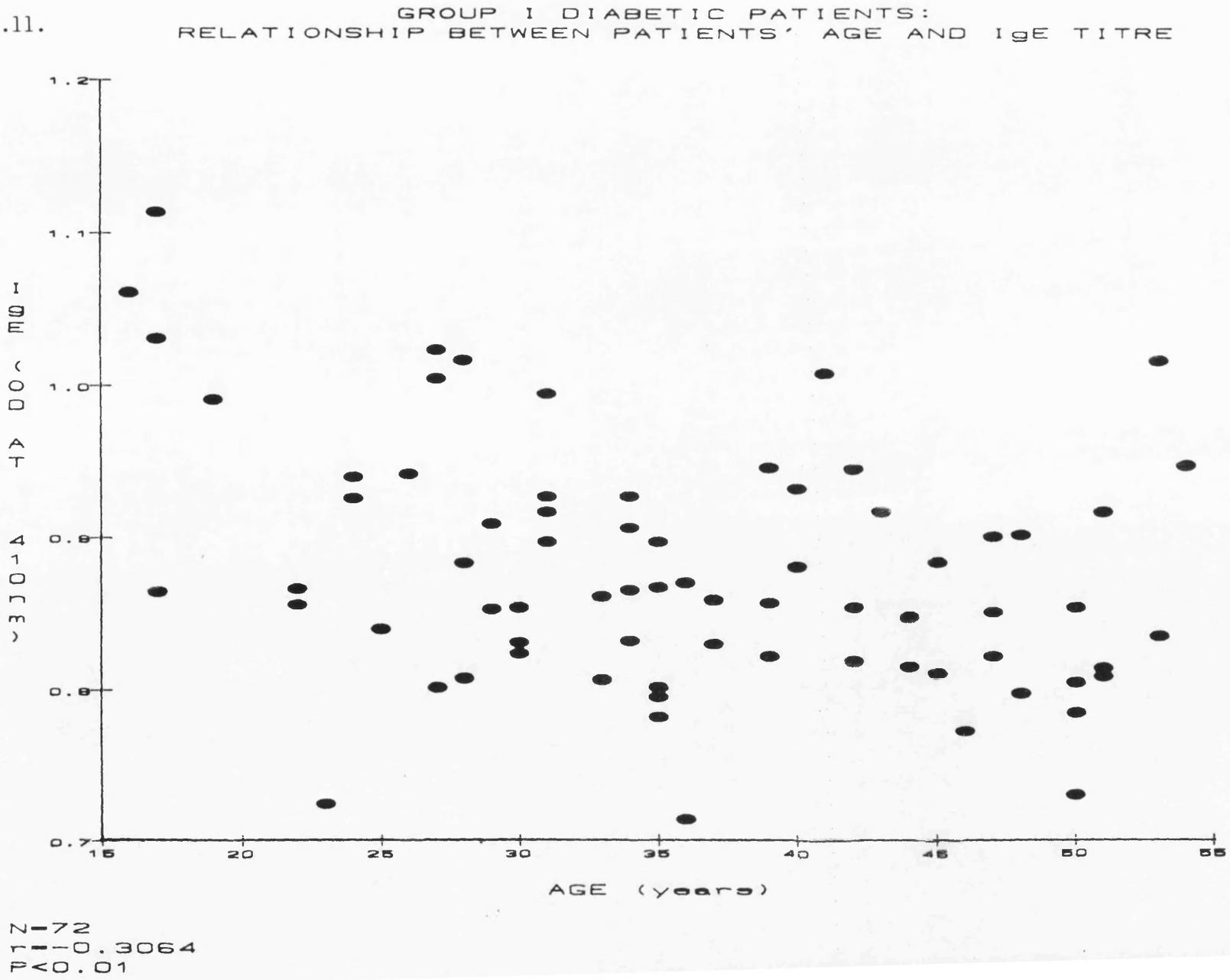
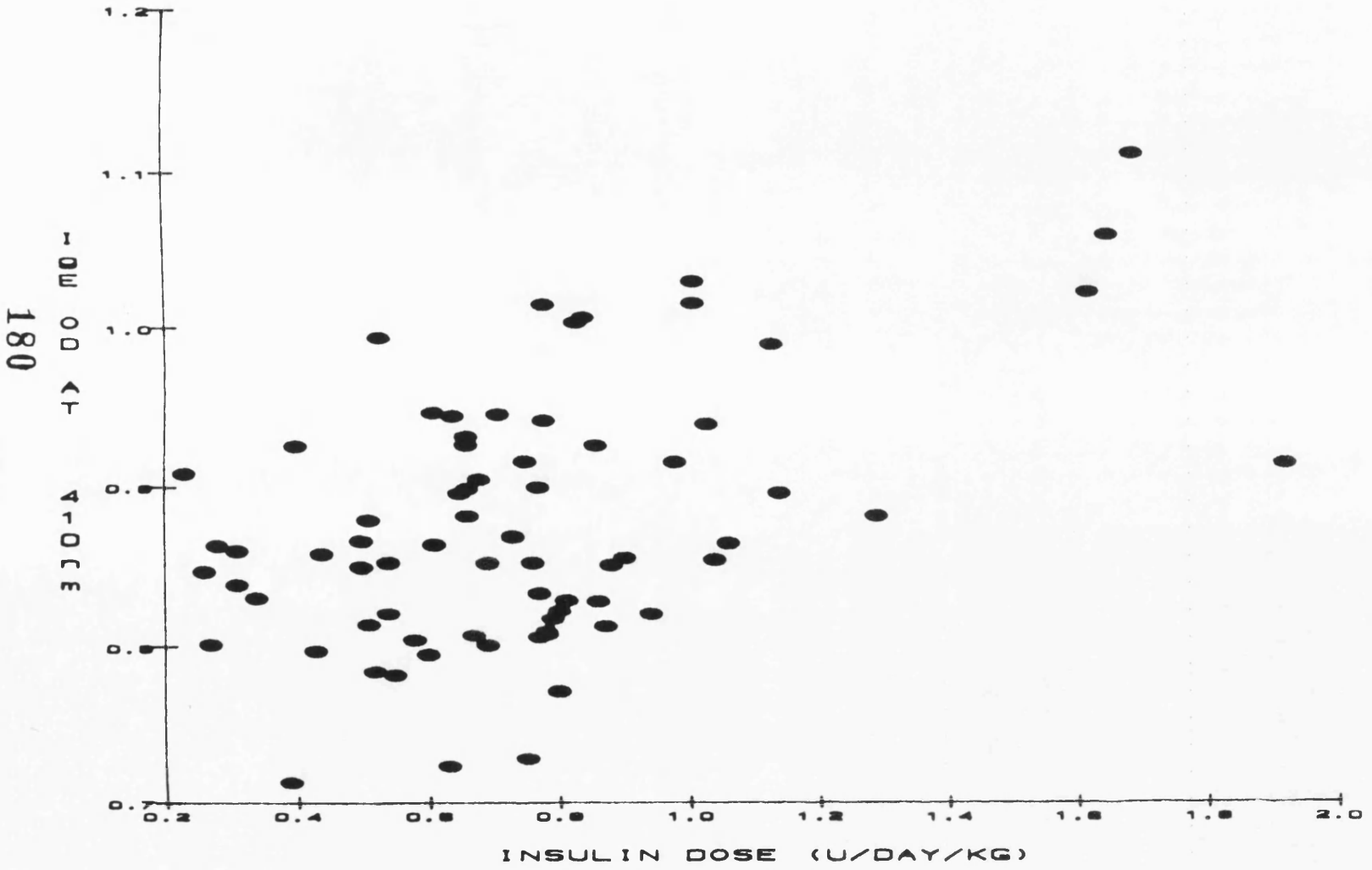
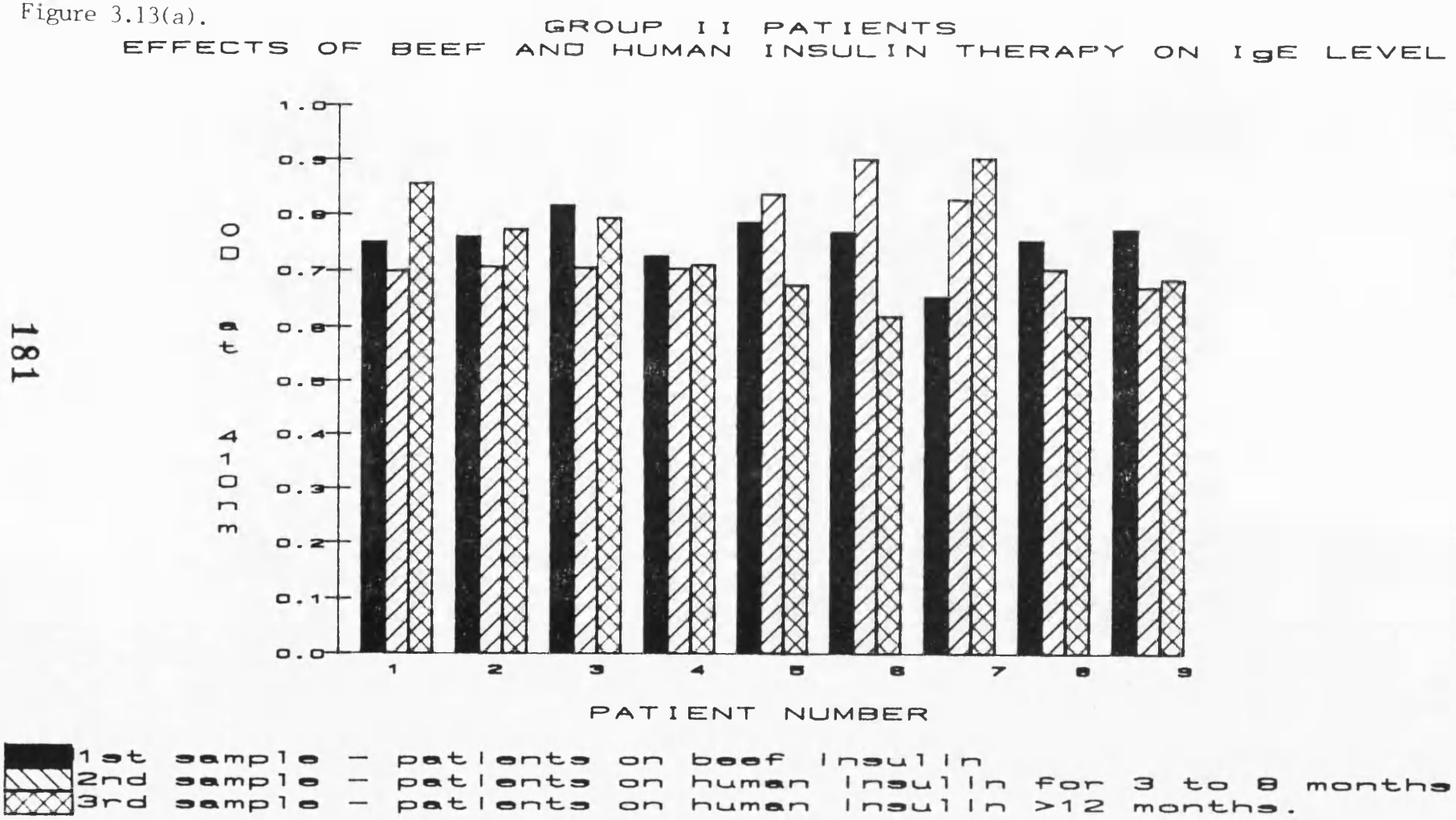

GROUP I DIABETIC PATIENTS:
RELATIONSHIP BETWEEN PATIENTS' AGE AND IgE TITRE
1.2
1.1
1.0
0.8
0.8
0.7
15
20
25
30
35
40
45
50
55
AGE (years)
N=72
r=-0.3064
P<0.01

Figure 3.12. RELATIONSHIP BETWEEN PATIENTS' DAILY INSULIN DOSE REQUIREMENT AND IgE TITRE



N=72
r=0.3577
P<0.01

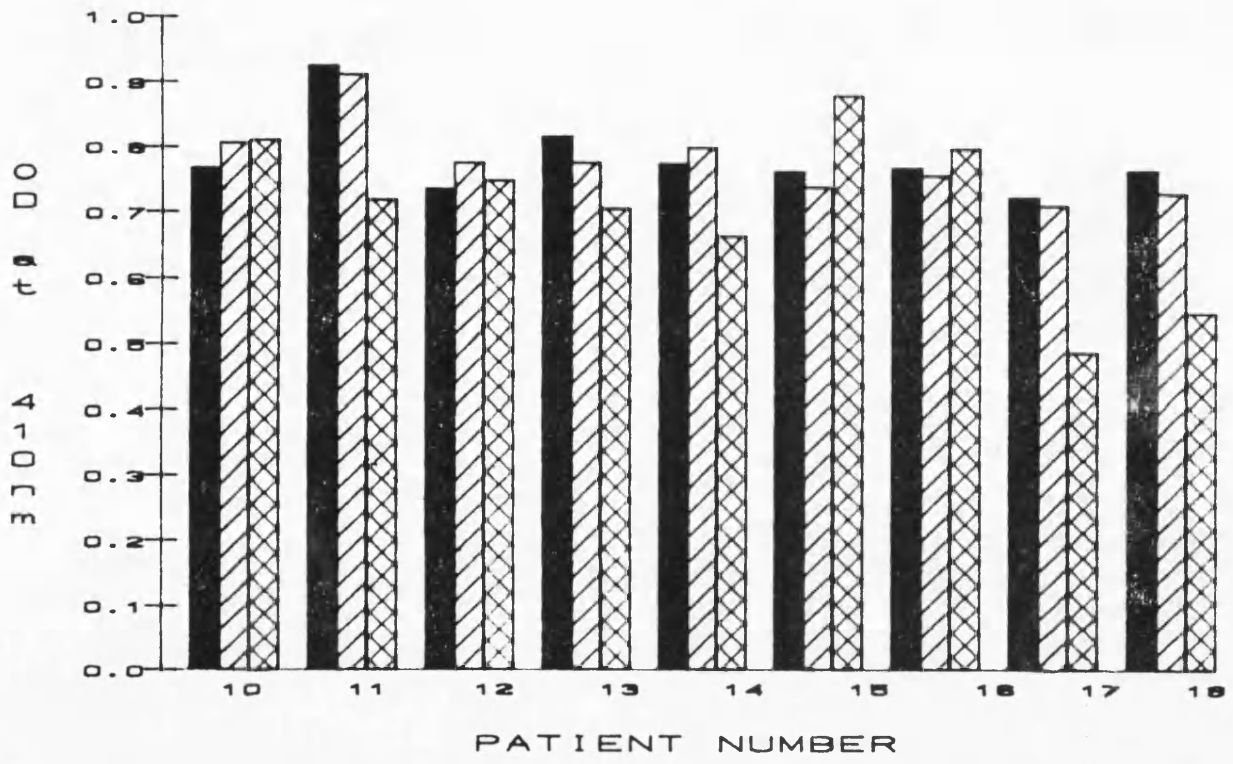
Figure 3.13(a).



Continued....

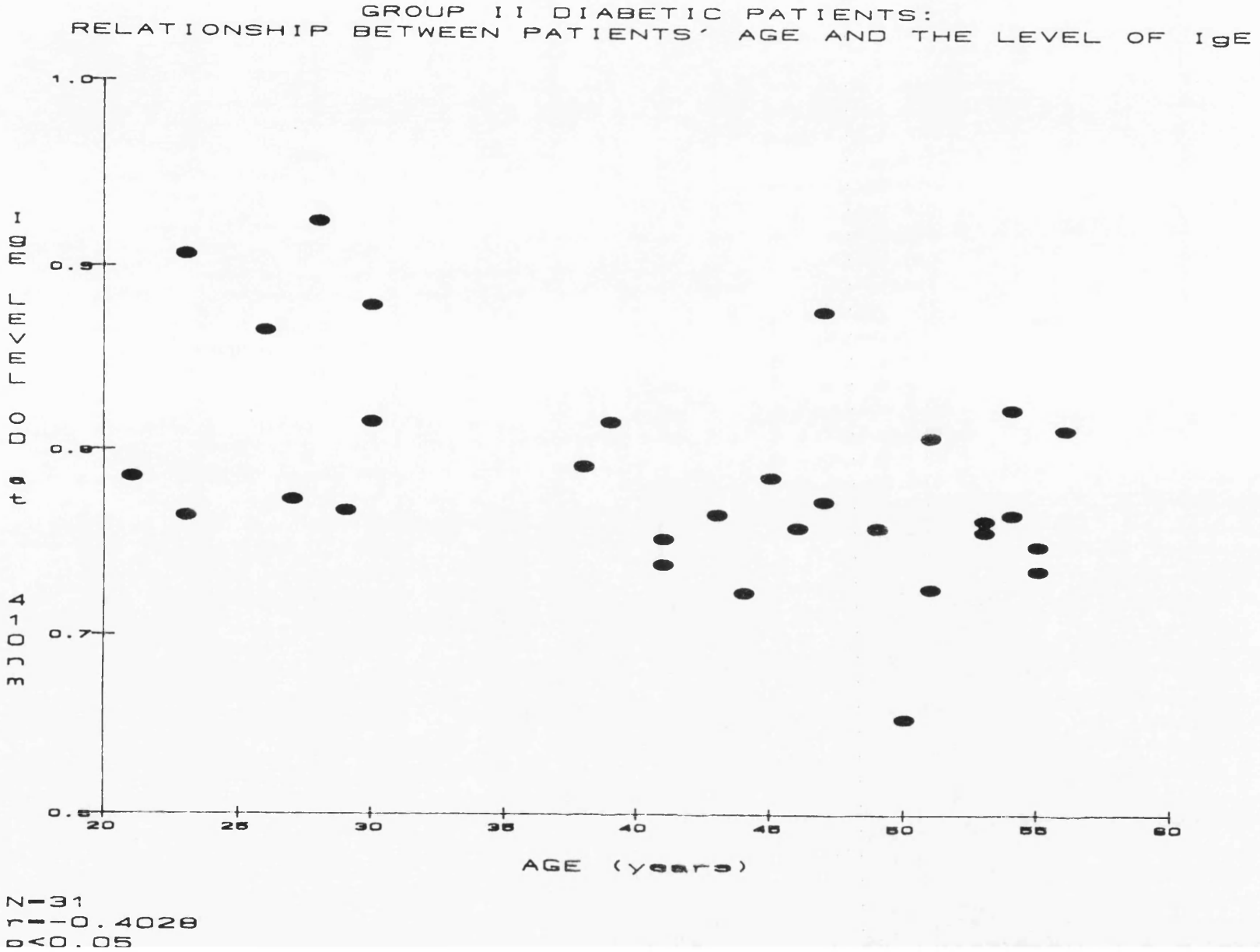
Figure 3.13(b).

GROUP II PATIENTS
EFFECTS OF BEEF AND HUMAN INSULIN THERAPY ON IgE LEVEL
continued....



1st sample - patients on beef insulin
2nd sample - patients on human insulin for 3 to 8 months
3rd sample - patients on human insulin for >12months.

Figure 3.14.


GROUP II DIABETIC PATIENTS:
RELATIONSHIP BETWEEN PATIENTS' AGE AND THE LEVEL OF IgE
1.0
0.9
0.8
0.7
0.6
20
25
30
35
40
45
50
55
60
AGE (years)
N=31
r=-0.4028
p<0.05

Interleukin-2 (IL-2) In Sera Of Diabetic And Control Subjects.

The concentration of IL-2 in sera of 15 Group I diabetic patients and 8 non-diabetic control subjects was determined using a human IL-2 ELISA kit (Intertest-2) purchased from Genzyme Cooperation, Boston, MA, USA. Table 3.19. gives the concentration of IL-2 found in the sera of individual subjects as well as the group means + SD for diabetic patients and controls.

Table 3.19.

Concentration of IL-2 in sera of Group I diabetic patients and non-diabetic control subjects:

Diabetic Patient	IL-2($ng.ml^{-1}$)	Control Subjects	IL-2 ($ng.ml^{-1}$)
No. 1	0.550	No. 1	0.340
2	0.019	2	2.200
3	0.123	3	0.840
4	0.017	4	1.500
5	0.420	5	1.125
6	0.058	6	1.090
7	0.330	7	1.500
8	0.210	8	2.275
9	0.001		
10	0.410		
11	0.550		
12	0.015		
13	1.150		
14	1.500		
15	0.520		
N	15		8
mean	0.392		1.359
SD	0.436		0.657
median	0.330		1.310

N= number of patients; No. = patient number; SD = standard deviation.

The concentration of IL-2 in sera of diabetic patients was found to be significantly lower than in sera of control subjects (Mann-Whitney U = 14; z = 2.97; P = .03).

3.6. DISCUSSION

With the recent advances in the production of immunochemicals which are more sensitive and specific than those used previously, it has been possible to design an ELISA with optimum conditions for the detection of anti-insulin antibody. The use of automated wash procedures and direct analysis of data by computer, reduces operator error to a minimum level. However, ELISA has one major drawback, insulin-coated plates are not suitable for studying the species specificity of antibody binding as there is much cross reactivity. Nevertheless, significant differences in the levels of anti-human, anti-pork and anti-beef insulin antibodies are reported in this study.

The data presented here largely confirm and extend previously published reports concerning anti-insulin antibody levels in sera of diabetic patients treated with human insulin (Group I) or beef insulin (Group II).

The anti-insulin antibody concentrations of sera of both Group I and Group II diabetic patients were found to be significantly higher than that of non-diabetic controls (which representa non-specific binding of antibody to insulin coated plates). Approximately 55% of 76 diabetic patients on human insulin therapy, possessed anti-insulin antibody, although the concentrations were quite low.

Similar results are quoted by other workers (Wilson, et. al., 1985; Di Mario,et al., 1986; Rogala, et. al., 1986; Lunetta, et. al., 1986). It is important to note at this point that almost all of the patients in Group I were simultaneously administered with short-acting (Actrapid MC and Humulin Soluble) and intermediate acting insulins (zinc suspension - Monotard, Ultratard and isophane insulins - Insulatard, Humulin). Since it is well established that zinc-insulin crystals may cause a significant increase in insulin immunogenicity (Galloway & Bressler, 1978; Diem, et. al., 1982; Nell, et. al., 1985) such therapeutic regimes may explain the high percentage of Group I diabetic patients with anti-insulin antibody.

For example, Lunetta, et. al., (1986) and Reeves et. al., (1984) found that the immunogenicity of short-acting monocomponent insulin preparations (both human and pork) was negligible. Patients administered with zinc-suspension preparations, however, gave a significant antigenic response. Unfortunately, in this study, it was not possible to assess the effects of zinc-insulin therapy on the level of anti-insulin antibody produced because only one patient of the 76 studied, was under Actrapid MC therapy alone. He was a 43 year old man (patient number 1; Table 3.3) who showed considerable insulin resistance (he was administered >200 units per day, by means of a continuous infusion insulin pump). His

serum anti-human insulin concentration was found to be 25.88 ug.ml^{-1}, which is a low positive antibody response according to the classifications used in this study.

It is interesting to note that all four Group I patients (patient numbers 6, 19, 30 and 32; Table 3.3) who produced high levels of anti-insulin antibody (>88.5 ug.ml^{-1} of serum) had at some time in their diabetic life been administered PZI beef insulin. Two of these four patients had diabetes retinopathy and one showed insulin allergy. It appears that the treatment with conventional insulin preparations can cause complications which are not necessarily reduced if the patient is transferred to human insulin. Patients who have been given human insulin from the onset of insulin therapy (8 patients) showed no or very low levels of insulin antibodies, thus implying that if human insulin is administered from the onset of insulin therapy, it is less immunogenic than conventional insulins.

The residual immunogenicity of therapeutic preparations of human insulin may also be due to molecular alterations which are thought to develop during manufacture, storage or following injection. For example, dimerisation or deamidation of the insulin molecule may take place which would create new determinants recognized by T- and B-cells.

The influence of HLA genotype on the level of insulin antibody is well documented (McEvoy, et. al., 1986;

Almer, et. al., 1985; Asplin, et. al., 1984). However, since it was not within the scope of this study to determine the patients' genotype, it's influence can not be ruled out as an explanation for the results obtained.

It has been supposed that anti-insulin antibodies are only produced against the variable residues in the therapeutically administered insulin molecule. However, a large proportion of the antibodies produced in response to the administration of heterologous insulin (beef and pork) is reactive with determinants present on the homologous molecule. i.e. autoreactive antibodies are produced (Reeves, 1986). This may explain the high degree of correlation observed between the three species of anti-insulin antibodies (i.e. human, pork and beef). The number of possible antibodies produced in response to insulin is vast, over 115 types has been identified using monoclonal antibodies which recognize unique sites on the insulin molecules (Schroer et. al., 1983). Most of these recognize epitopes present in all three species of insulin due to the high degree of homology between them. Insulin antibodies can bind to almost any part of the insulin surface and their chief metabolic effect is to withdraw significant amounts of insulin within the vascular compartment.

The data outlined in this study do not confirm any effect of age, sex, duration of disease, duration of insulin

treatment, or the daily insulin dose on the level of anti-insulin antibody concentration. Since Group I diabetic patients do not include the very young who are reported to produce higher levels of antibody (Andersen, 1972), and the very old (age range of Group I patients was 16 to 55 years), it may explain the lack of correlation between age and antibody response. It is well established that most long-standing, insulin treated diabetics are insulin resistant (Gray,et. al., 1985). Such insulin resistance may be a function of prevailing degree of metabolic control and duration of diabetes, and is unrelated to the presence of insulin antibodies when insulin action is assessed under steady state conditions (Gray, et. al., 1985). This observation accords with the failure to show a direct relation between daily insulin dose and insulin antibody binding.

There was, however, a significant correlation between the level of anti-insulin antibody and diabetic control as determined by glycosylated haemoglobin level. This was especially significant in the level of anti-beef insulin antibody.

Haemoglobin A_{1c} (HbA_{1c}) is the major glycosylated haemoglobin present in red blood cells of healthy adults and diabetic patients. It is the product of a chemical reaction between glucose and the N-terminal valine of the beta chain of haemoglobin Ao. The concentrations of HbA_{1c}, as well as those of three minor haemoglobins are

elevated in diabetic patients. The concentration of HbA1 (HbA_{1a} + HbA_{1b} + HbA_{1c} = HbA1) is mainly dependent on the prevailing plasma glucose level, and it is commonly used as means of assessing metabolic control in diabetic patients. Results outlined in the present study show that patients with stable diabetic control (stable HbA1 value, <11%, determined over one year period) have significantly higher levels of anti-insulin antibody than those with unstable control (>13% HbA1, and frequent changes in insulin dose).

Insulin binding antibodies were found to be present in the serum of approximately 50% of the patients studied. They are said to act as insulin transporting proteins (Dixon, et. al., 1975). Insulin entering the vascular space from its injection depot site is thought to combine with insulin antibody so that only a proportion of the insulin remains free in serum to exert its physiological action. When the depot site is exhausted or when the flux of insulin into serum is inadequate, the antibody-insulin complex dissociates and insulin is released. This prevents the concentration of insulin falling to low levels (Dixon et. al., 1975). This 'buffering effect' of anti-insulin antibodies has the effect of damping oscillations in free insulin levels and thereby leads to stable diabetic control. Unstable diabetes results when insulin antibodies do not act as a buffer, either because the serum concentration of

anti-insulin antibody is low or the antibody avidity for insulin is high (Dixon, et. al., 1975).

The hypothesis that diabetic stability may be associated with the presence of anti-insulin antibodies is also suported by other workers (Dixon, et. al., 1975; Gray, et. al., 1981; Gray, et. al., 1985; McEvoy, et. al., 1986; Walford, et. al., 1982; Keilaker, et. al., 1982; Keilaker, et. al., 1986), although Asplin et. al., (1978) and Goldman et. al., (1979) failed to find such a relationship.

Anti-Insulin IgG Subclasses.

Human IgG consists of four subclasses based on antigenic differences in their heavy polypeptide chains. The IgG subclasses are isotypes of IgG, and their distribution pattern is inherited. Each subclass contains special Gm factors. The IgG subclasses are found in different proportions in normal serum with the relative concentrations for IgG1 - 60 to 70%, IgG2 - 14 to 20%, IgG3 - 4 to 8%, and IgG4 2 to 6% (Steinberg et. al., 1973). The IgG subclass distribution, expressed in terms of proportion of total IgG in the sera of non-diabetic control subjects was found to be 59.9% IgG1, 18.3% IgG2, 9.2% IgG3 and 12.6% IgG4. The results obtained in this study were similar to that quoted by Steinberg et. al., although the level of IgG4 was found to be highr than expected. This may be due to the high avidity of

anti-IgG4 monoclonal antibody used in this study.

The mean anti-insulin IgG subclass distribution in insulin-dependent diabetic patients were found to be 47% IgG1, 13% IgG2, 28% IgG3 and 11% IgG4. In most patients IgG1 was the predominant subclass, second most abundant subclass being IgG3. In 5 out of the 42 patients' sera tested, IgG3 was the most dominant subclass. This is most interesting in view of the fact that secondary systemic antibody responses to protein and viral antigens are predominantly of the IgG1 and IgG3 subclasses with the proportions varying with the antigen and between individuals (Shakib, 1986).

Anti-insulin IgG2 has been reported to be deficient and/or absent in diabetic patients by Koch et. al., (1986) and Oxelius (1984). These results may be due to the use of incorrect anti-IgG2 monoclonal antibodies. For example, Koch et. al. used BAM 10 - clone GOM1. This particular clone was also used in this study to determine the anti-insulin IgG2 subclass distribution and similar results to Koch et. al., were obtained. However, further analysis of the clone using normal serum revealed its inappropriateness for the ELISA technique as very low levels of anti-IgG2 were obtained in all the sera from control subjects. Jefferis, et. al., (1985), also came to similar conclusions. They found that only two of the nine anti-IgG2 monoclonal antibodies commercially available, had high avidity for IgG2. Therefore, a more

appropriate clone (HP6014) was used for reassessment of the anti-insulin IgG subclass distribution. Such methodological problems reflect the importance of proper assessment of reagents used for a particular ELISA system.

In four patients, IgG4 was as high as 30 percent or more. In these patients the high level of IgG4 may be associated with chronic antigenic stimulation (Aalberse, et. al., 1983; Shakib, 1986). These four patients' daily insulin dose requirement was among the highest (three of the four patients required >1.62 U/D/Kg; the fourth patient required 0.98 U/D/Kg). Interestingly, one of these patients, with 33.7% IgG4 (he required a daily insulin dose of 1.65 U/D/Kg), was a 16 year old boy who suffered from severe hypersensitivity to insulin at the injection site, i.e. he was allergic to insulin. Furthermore, a significant positive correlation between IgG4 and the patients' daily insulin dose requirement was observed which may reflect the effects of chronic antigenic stimulation.

Aside from insulin-dependent diabetes, haemophilia is another disease in the course of which a protein is frequently administered. It is worth noteing that the profile of anti-factor VIII immunization also shows an abnormal IgG distribution, since it is characterized by a higher than expected abundance of IgG4 (Shakib, 1986). Admittedly, coagulation factor VIII is a glycoprotein

whereas insulin does not contain any saccharidic components, but it is possible that proteins and glycoproteins induce an abnormal level of IgG4 and that this level is dose dependent.

Compared to IgG4, an inverse relationship was observed between insulin dose and IgG2. This phenomenon is less easily explained. IgG2 is reported to be deficient in several autoimmune diseases (Oxelius, 1984; Shakib, 1986), including type I diabetes (Koch et. al., 1986). In this study 8 of 42 patients investigated produced little or no IgG2. It would be interesting to determine whether lack of IgG2 is a reflection of the autoimmune process, i.e., patients with little beta cell function, require a higher insulin dose (Goldman et. al., 1979; Ludvigsson, 1984), and are unable to synthesise normal levels of IgG2. There was also an apparent decrease in IgG2 with duration of disease, but it just failed to be statistically significant ($r=-0.289$; $P=0.053$).

The relative percentage of IgG4 decreased with `age'. IgG4 is presumably harmless, it does not fix complement and may possibly be protective against complement-induced damage, this protective effect of IgG4 may be lost with age and could lead to many of the immunological complications associated with old age in diabetic patients. Further investigation of the relationship between IgG4 and diabetic complications is obviously necessary in order to validate this hypothesis.

Effects Of Transferring From Beef To Human Insulin Therapy

Approximately 48% of Group II diabetic patients possessed anti-human insulin antibodies and 32% possessed anti-beef insulin antibodies while they were undergoing beef insulin therapy. Significant differences in the level of anti-human and anti-beef insulin were observed in all three serum samples.

In the majority of Group II diabetic patients, the concentration of anti-insulin antibody was unaffected by the type of insulin therapy. High levels of antibody were seen in so few patients (4 of 31 patients studied) that statistical analysis was difficult, but it was evident that in those with very high antibody titer, there was a substantial decrease in anti-insulin antibody concentration when the patients were transferred from beef to human insulin therapy. It is possible that beef insulin is more immunogenic than human insulin in a selective number of patients who are sensitive to insulin due possibly to genetic factors. According to the overall results, however, the level of anti-beef insulin antibody significantly increased 12 months after the patients were transferred to human insulin. Such findings rather refutes the idea that beef insulin is more antigenic than human insulin due to the difference in amino acid sequence between homologous insulin and beef insulin. Other factors clearly play a role in the

antigenicity of the therapeutic insulin preparations, such as purity, additives such as zinc, proinsulin content, mode of administration and so on.

For example, Wilson, et. al., (1985) found that a reduction in proinsulin contamination of beef insulin to <1 ppm lowers antibody levels in patients previously "immunized" with a conventional beef insulin preparation. Highly purified beef insulin (Neuphane and Neusulin with very little proinsulin) had been administered to Group II diabetic patients. Therefore, the lack of significant reduction in insulin antibody titer when the patients were transferred to human insulins (Human Velosulin and Insulatard) may be due to the fact that highly purified beef insulin is not very immunogenic anyway (Wilson et. al., 1985).

Di Mario, et. al., (1986) also found that a switch to biosynthetic human insulin in patients already treated with highly purified insulins, (as is the case in Group II patients), did not modify the levels of anti-insulin antibodies. It is possible however, that with long-term use of human insulin, the patients may reveal positive immunological modifications, which was not detected in this short-term investigation of one year. For example, recent studies on the immunogenicity of human insulin has shown that the level of anti-insulin antibodies continues to decrease after 12 months following the transfer of patients to human insulin therapy (Chandraprasert &

Bunnag, 1985; Rogala et. al., 1986). Thus the antibody measured may in fact be anti-insulin antibody induced by beef insulin therapy prior to change to human insulin therapy. The level of *anti-human* insulin antibody in the 1st sample, may also be explained by the fact that antibodies induced by beef insulin cross react to a large extent with the human insulin molecule (Wilson et. al., 1985). Such a phenomenon may explain the the lack of reduction in antibody response when transferred to human insulin and a further year of follow up study is necessary on the same patients before a substantial lowering of anti-insulin antibody might be observed. It is important to note, however, that the level of anti-human insulin antibody produced by Group II patients (at all three occasions), did not differ significantly from that produced by Group I diabetic patients, most of whom have been on human insulin therapy for more than two years.

Because the number of patients studied (31 in Group II) was low the results outlined in this study is not necessarily representative of the effect of beef and human insulin therapy on the immune response to insulin. Other factors such as HLA type (Ludvigsson, 1984; Reeves et. al., 1984; Almer, et. al., 1985) probably play an important role. For example, patients with HLA-15, DR4 or DR3/4 phenotype have a high responder status, whilst patients with HLA-B8, or lacking B7, have a low responder

status to both beef and pork insulins. Patients possessing the HLA-DR3 phenotype respond normally to beef insulin, but are of low responder status to pork insulin (Sklenar, et. al., 1982).

Effects Of Beef And Human Insulin Therapy On IgG Subclass Distribution.

The relative percentage of anti-insulin antibody IgG3 and/or IgG4 subclasses increased in four of the 9 patients tested, but the overall distribution of any one subclass did not change significantly when the patients were transferred to human insulin therapy. The increase in antibodies of the IgG3 and IgG4 subclasses in some patients is difficult to account for without knowledge of the clinical background of Group II patients. The HLA type of these patients may also be of great interest in that the HLA type may influence the IgG subclass distribution. It is speculated that human insulin therapy induces more IgG3 and IgG4 than beef insulin therapy.

IgM And IgE

A qualitative analysis of IgM and IgE present in serum of diabetic patients were performed. The levels of anti-insulin IgE and IgM were found to be too low to detect using the assay system availabe at the time of study. The lack of a suitable anti-insulin IgE or IgM standard also made it difficult to quantitate these two isotypes of anti-insulin antibodies. Therefore the total IgE and IgM protein levels were determined. IgM was found to be significantly lower in diabetic patients than in non-diabetic controls.

IgE is associated with homocytotropic antibody activity in man. It is the main class of antibody produced in response to allergic reactions (Shakib, 1986). The level of IgE in diabetic patients was not found to be significantly different from that of non-diabetic controls. This suggests that insulin allergy probably is not a common feature amoung the patients investigated in this study.

The level of IgE protein was found to decrease with age and increased with the patients' daily insulin dose intake. The high level of IgE in patients requiring high insulin dose may reflect a degree of insulin allergy in some patients. For example, one patient with insulin allergy, which manifested as lumps formed at the injection site, produced a high level of IgE compared to

most diabetics studied. The decreased level of IgE production with age may be a side effect of the ageing process in general.

CHAPTER IV

RELATIONSHIP BETWEEN CELLULAR AND HUMORAL IMMUNE RESPPONSE TO INSULIN.

4.1. RESULTS

4.1.1. *Relationship between cellular immune response to insulin and anti-insulin IgG.*

The possible relationship between cellular (both SI and % suppression) and humoral immune response to insulin in Group I diabetic patients was investigated using Spearman's Rank correlation coefficient test. The results are tabulated in Tables 4.1. and 4.2.

Table 4.1. and 4.2. show that in most cases the correlation between cellular response (stimulation index & % suppression and total anti-insulin IgG concentration was not significant. There was, however a significant correlation between IgM and the stimulation index in the presence of $10ug.ml^{-1}$ human insulin and $10ug.ml^{-1}$ beef insulin (see Figures 4.1. and 4.2. respectively). Figure 4.3. shows that the level of IgE also correlated with SI in the presence of $100ug.ml^{-1}$ pork insulin. Although the correlation between % suppression at 10 $ug.ml^{-1}$ human insulin and IgE was not significant, plotting these two parameters (Figure 4.4.) shows a clear cut positive relationship between IgE and % suppression. The relationship became significant if the two patients who

gave very high negative % suppression values (-315% and -729.8%) were removed from the analysis ($r=0.356$; $P<0.05$). The corresponding levels of IgE were 0.947 and 0.880 (O.D. at 410nm) respectively.

In Table 4.3., the relationship between total anti-insulin IgG, anti-insulin IgG subclass antibodies, IgE and IgM are tabulated. Only IgG4 showed a significant correlation with IgE; an increase in IgE seems to be associated with a rise in IgG4, Figure 4.5. illustrates this relationship. No other correlation was observed.

Table 4.1.

Relationship between cellular (SI) and humoral immune response to insulin in Group I diabetic patients. Spearman's Rank Correlation Coefficient (r_s):

	Cellular Immune Response To Insulin (SI)					
	H10	H100	P10	P100	B10	B100
N	22	22	35	35	35	35
Antibody Response						
anti-HI Ab	-0.013	-0.040				
anti-PI Ab			-0.037	-0.047		
anti-BI Ab					-0.122	-0.158
N	39	39	59	61	60	61
IgM	-0.352 P<0.05	-0.274	-0.071	-0.134	-0.274 P<0.05	-0.118
IgE	-0.066	-0.198	-0.216	-0.286 P<0.05	-0.097	-0.119

N=number of patients. SI=stimulation index. Ab=antibody. HI=human insulin; PI=pork insulin; BI=beef insulin at 10 and 100ug.ml^{-1}. SI:Type and concentration of insulin in culture: H=human; P=pork; and B=beef; H=human insulin at 10 and 100ug.ml^{-1}

Table 4.2.

Relationship between % suppression and antibody concentration. Spearman's Rank Correlation Coefficient (r_s) values:

	% Suppression					
	H10	H100	P10	P100	B10	B100
N	22	22	35	35	35	35
Antibody Response						
anti-HI Ab	0.002	0.139				
anti-PI Ab			0.101	0.102		
anti-BI Ab					0.065	0.058
N	39	39	59	61	60	61
IgM	-0.014	0.276	-0.068	0.021	0.134	0.112
IgE	0.314*	-0.050	0.050	-0.053	0.040	-0.100

*Type and concentration of insulin in culture: H=human; P=pork; B=beef insulin at 10 and 100ug.ml^{-1}. Ab=antibody. *=P<0.053.*

Table 4.3.

Relationship between total IgG, IgG subclasses and IgM & IgE. Spearman's Rank Correlation Coefficient (r_s) values:

	Anti-Insulin IgG			Anti-Insulin IgG Subclasses			
	anti-HI	anti-PI	anti-BI	IgG1	IgG2	IgG3	IgG4
N	76	76	76	41	41	41	41
IgM	-0.08	-0.03	-0.021	-0.20	-0.14	0.17	-0.07
IgE	-0.05	-0.03	0.04	-0.04	-0.14	-0.15	0.441 P<0.01

N=number of patients.

Figure 4.1.

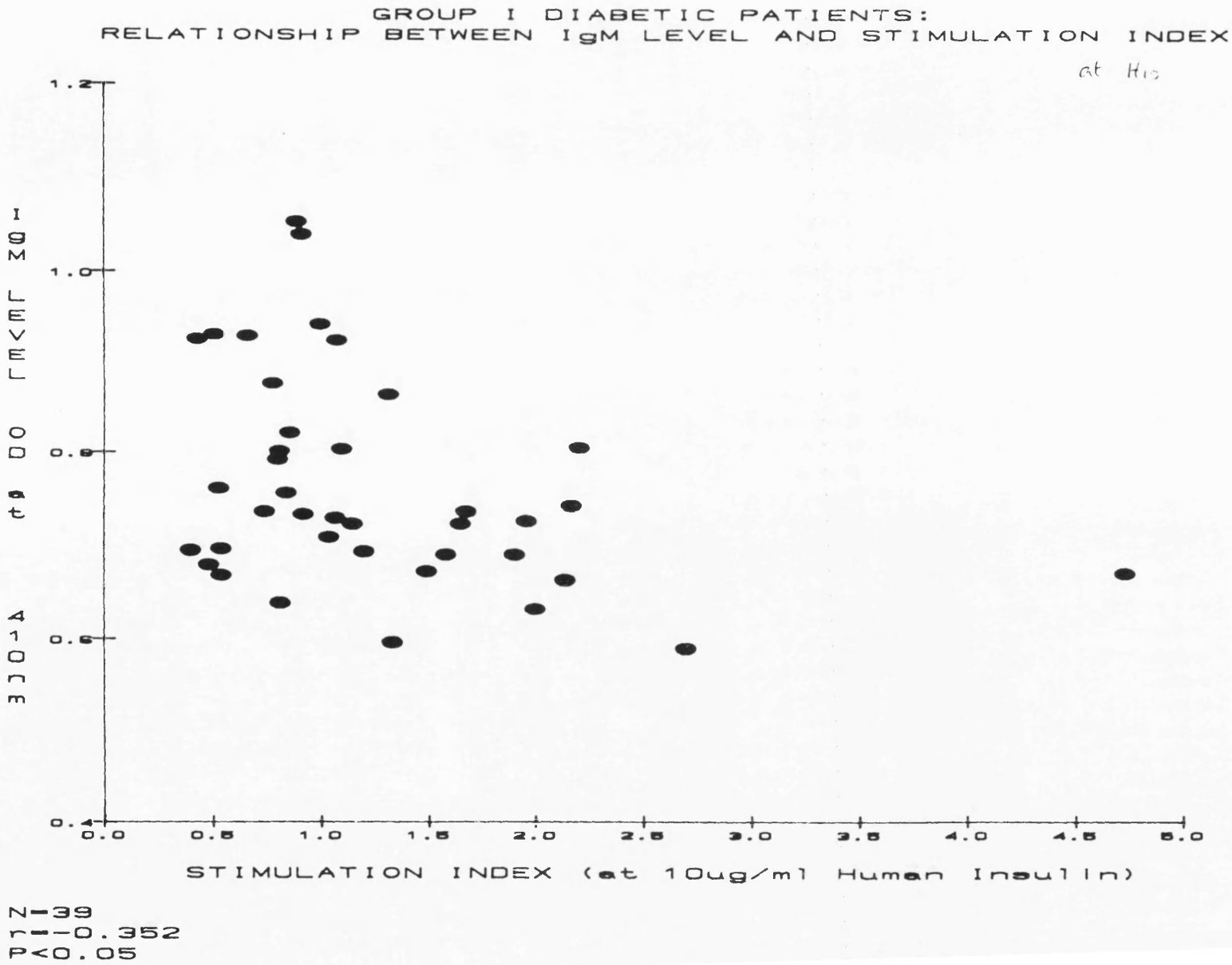

GROUP I DIABETIC PATIENTS:
RELATIONSHIP BETWEEN IgM LEVEL AND STIMULATION INDEX
at His
IgM LEVEL (mg %)
1.2
1.0
0.8
0.6
0.4
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
STIMULATION INDEX (at 10ug/ml Human Insulin)
N=39
r=-0.352
P<0.05

Figure 4.2.

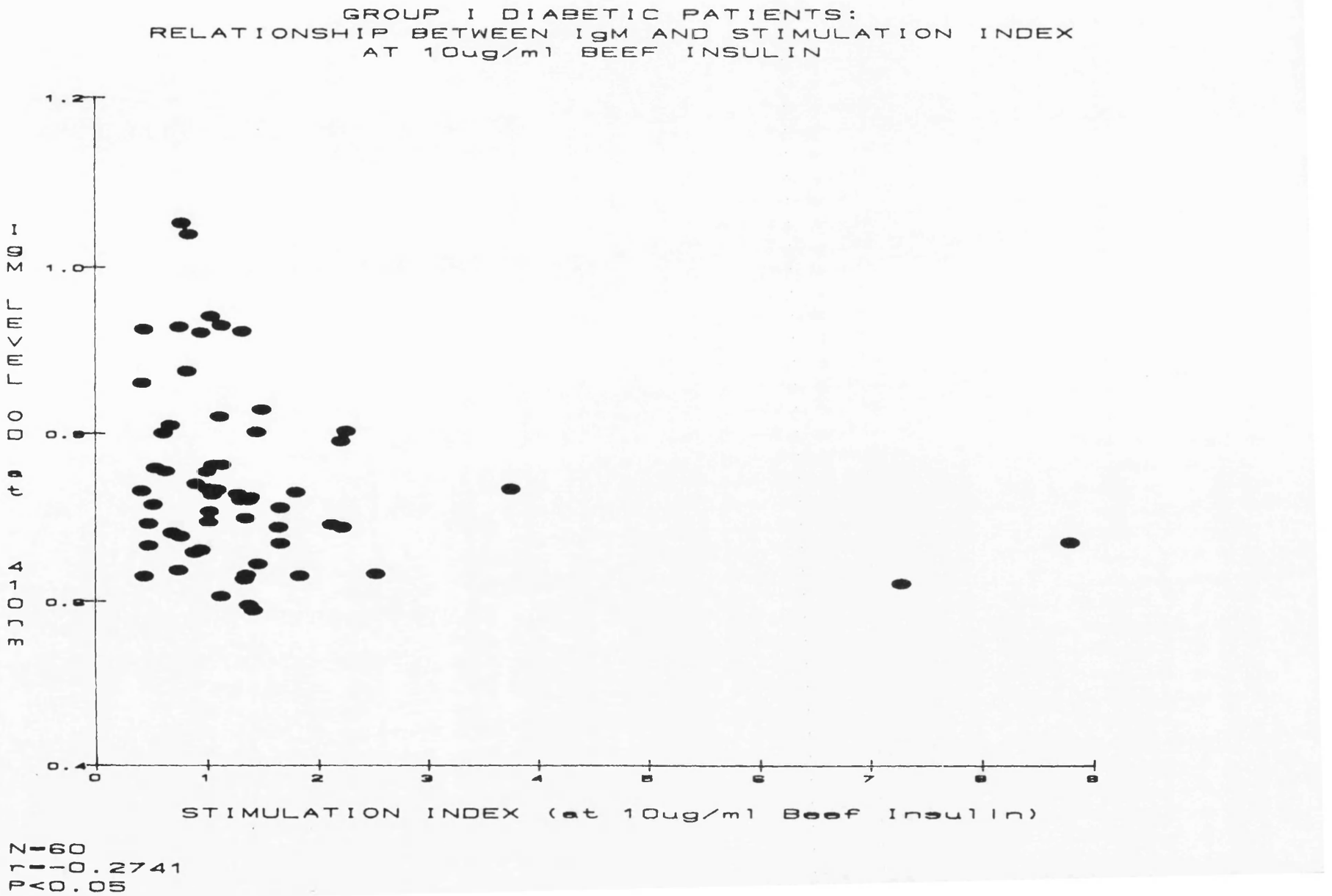

GROUP I DIABETIC PATIENTS:
RELATIONSHIP BETWEEN IgM AND STIMULATION INDEX
AT 10ug/ml BEEF INSULIN
IgM LEVEL at 1:40 DILUTION
1.2
1.0
0.8
0.6
0.4
0
1
2
3
4
5
6
7
8
STIMULATION INDEX (at 10ug/ml Beef Insulin)
N=60
r=-0.2741
P<0.05

Figure 4.3.

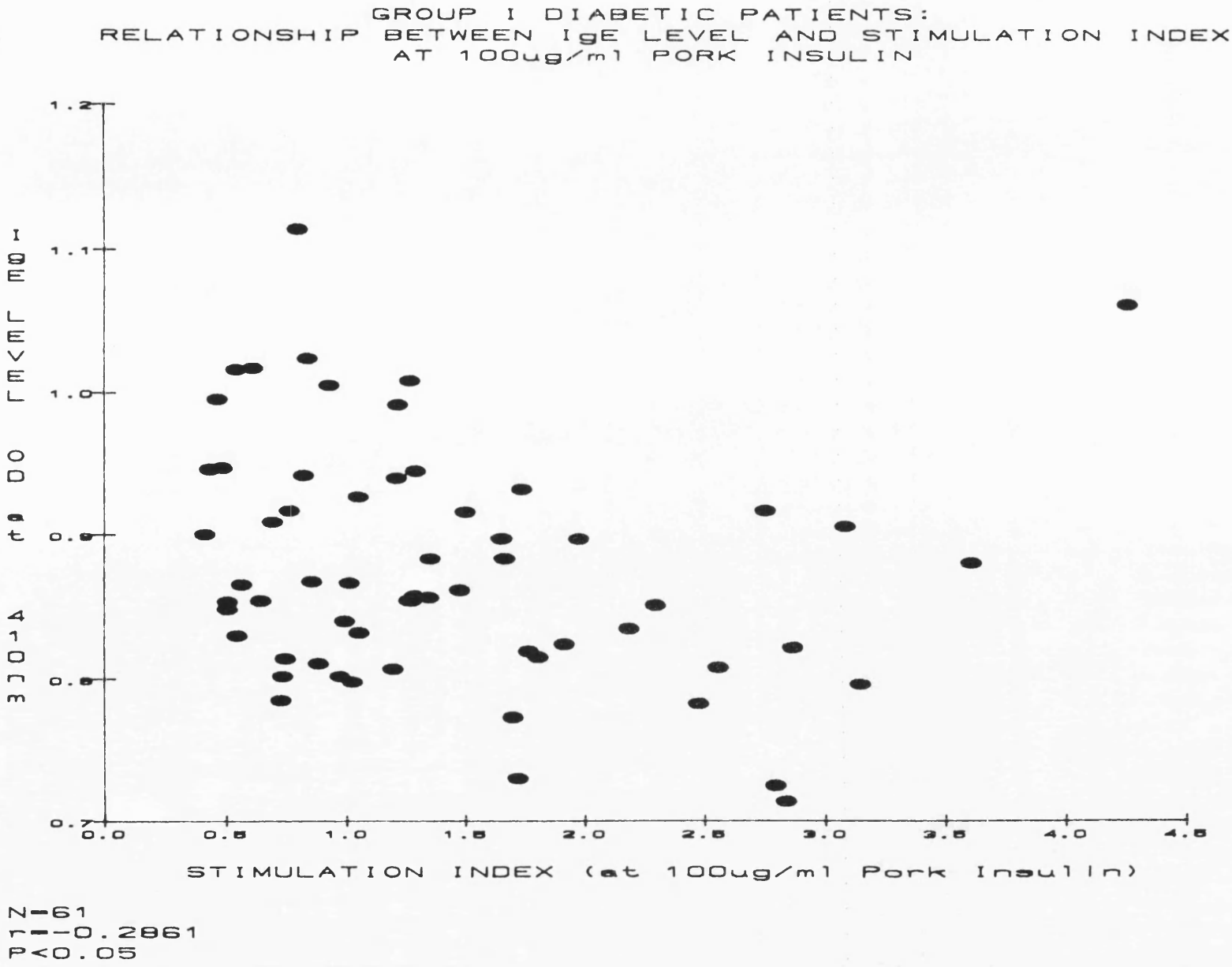

GROUP I DIABETIC PATIENTS:
RELATIONSHIP BETWEEN IgE LEVEL AND STIMULATION INDEX
AT 100ug/ml PORK INSULIN
IgE level to DO 3704
1.2
1.1
1.0
0.9
0.8
0.7
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
STIMULATION INDEX (at 100ug/ml Pork Insulin)
N=61
r=-0.2861
P<0.05

Figure 4.4.

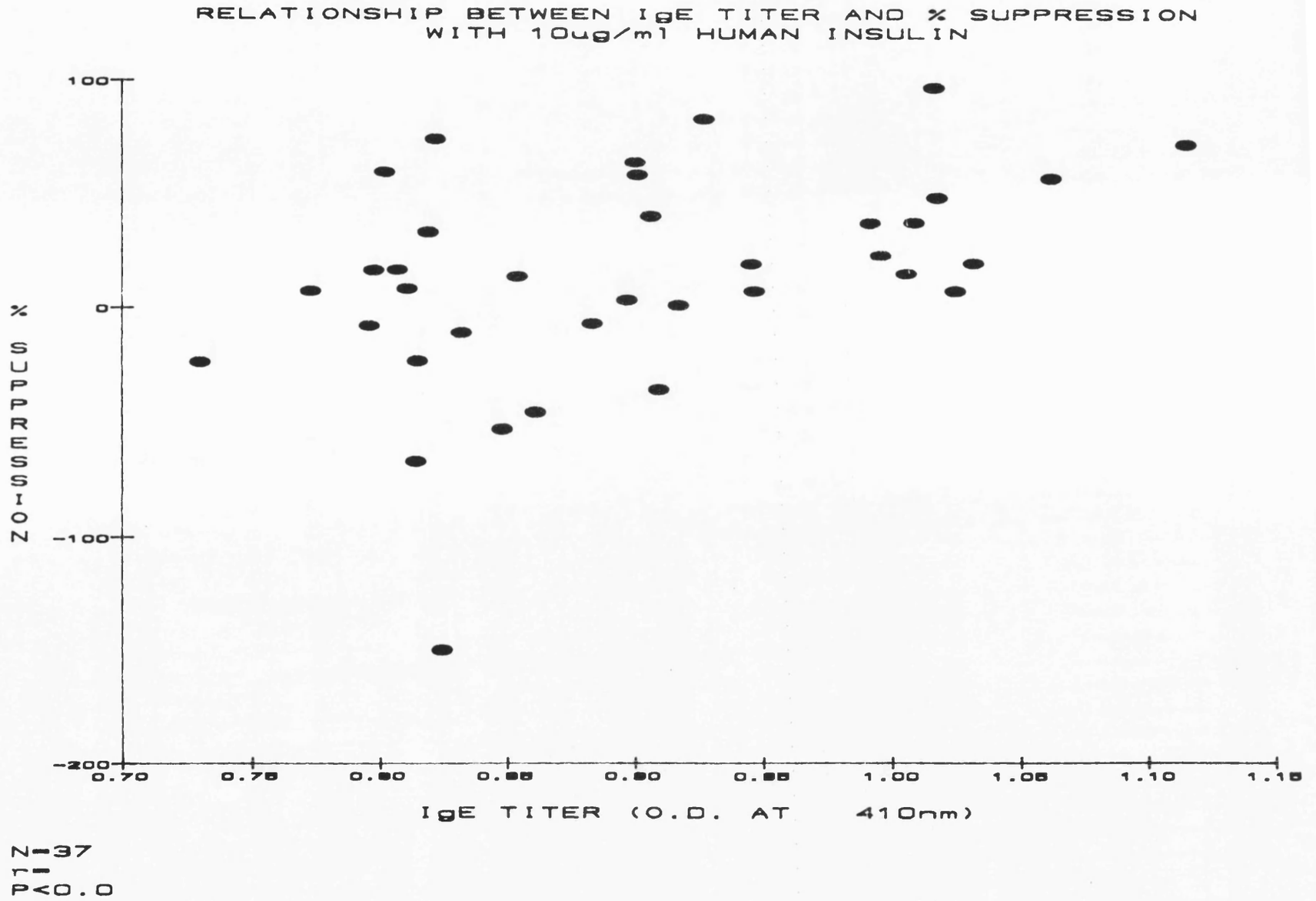
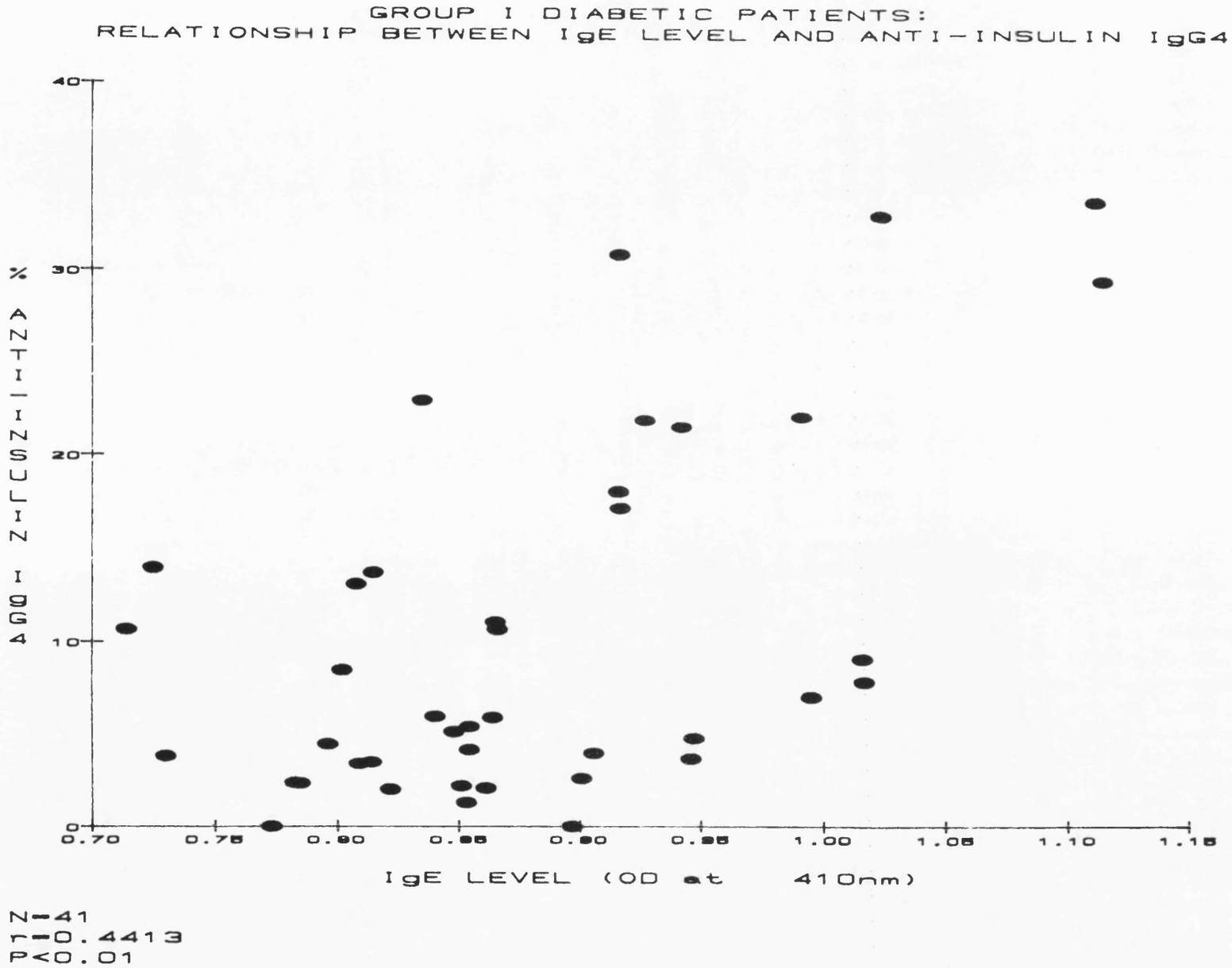

RELATIONSHIP BETWEEN IgE TITER AND % SUPPRESSION
WITH 10ug/ml HUMAN INSULIN
100
0
-100
-200
% SUPPRESSION
0.70
0.75
0.80
0.85
0.90
0.95
1.00
1.05
1.10
1.15
IgE TITER (O.D. AT 410nm)
N=37
r=
P<0.0

Figure 4.5.


GROUP I DIABETIC PATIENTS:
RELATIONSHIP BETWEEN IgE LEVEL AND ANTI-INSULIN IgG4
% ANTI-INSULIN IgG4
40
30
20
10
0
0.70
0.75
0.80
0.85
0.90
0.95
1.00
1.05
1.10
1.15
IgE LEVEL (OD at 410nm)
N=41
r=0.4413
P<0.01

4.1.2. *Relationship Between Cellular Immune Response To Insulin And Anti-Insulin IgG Subclass Distribution.*

The relationship between SI (and % Suppression) and IgG subclass distribution was analysed. Tables 4.4. and 4.5. shows the Spearman's rank correlation coefficient values.

The relationship between cellular immune response to insulin and anti-insulin IgG subclasses was not significant, except in the case of IgG4 which increased with a rise in % suppression in the presence of 10 $ug.ml^{-1}$ human insulin. Figure 4.6 illustrates this relationship.

Table 4.4.

Relationship between cellular immune response (SI) to insulin and anti-insulin IgG subclass distribution. Spearman's Rank Correlation Coefficient (r_s):

	Cellular Immune Response To Insulin (SI)					
	H10	H100	P10	P100	B10	B100
N	22	22	35	35	35	35
Anti-Insulin IgG Subclasses						
anti-IgG1	0.292	0.152	0.042	0.221	0.113	0.243
anti-IgG2	-0.063	-0.103	0.040	-0.187	-0.247	-0.264
anti-IgG3	-0.285	-0.095	-0.293	-0.279	-0.296	-0.123
anti-IgG4	-0.165	-0.095	-0.065	0.110	-0.153	-0.177

N=number of patients. Type and concentration of insulin in culture: H=human; P=pork; and B=beef insulin at 10 and 100ug.ml^{-1}. SI=stimulation index.

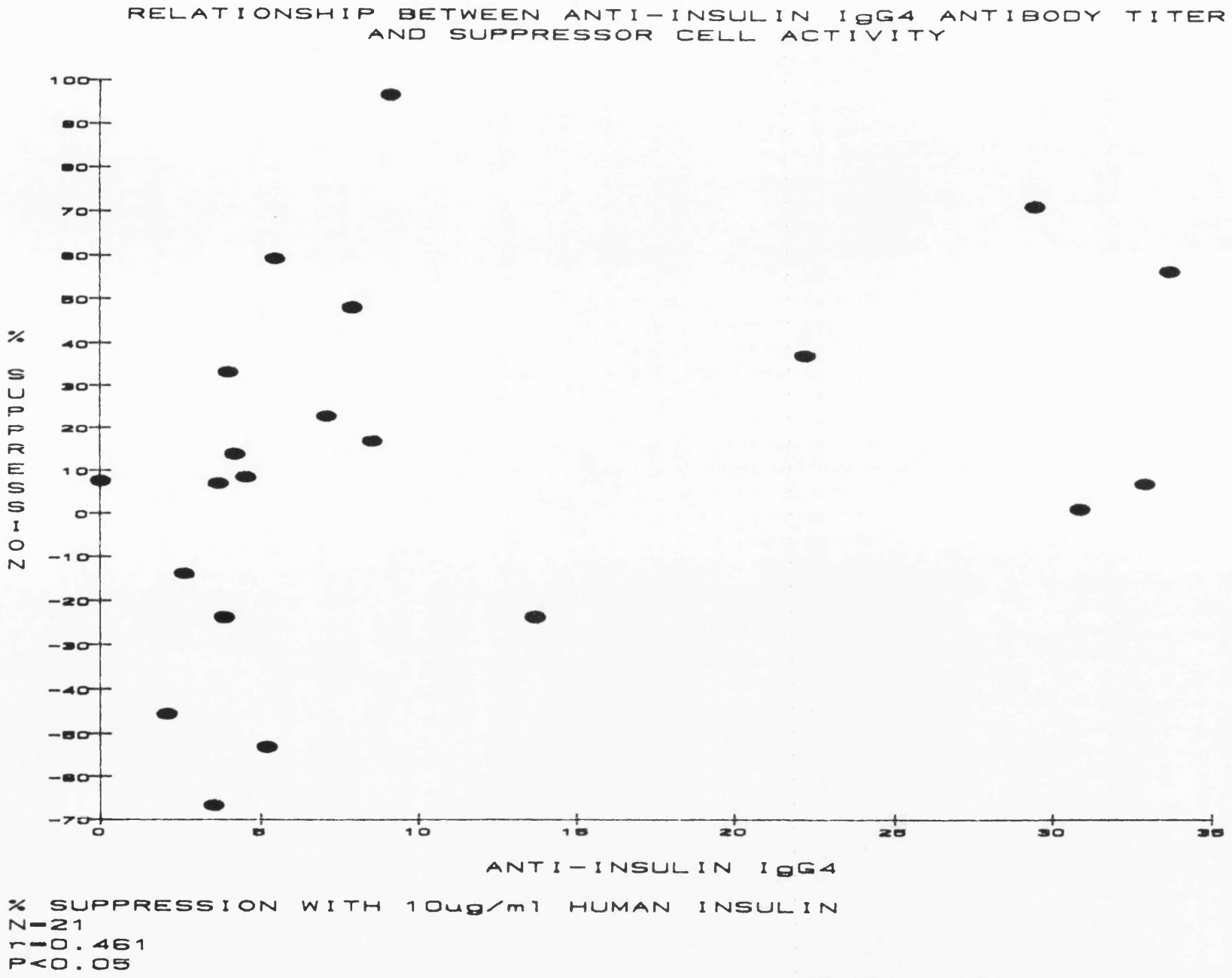
Table 4.5.

Relationship between % suppression and anti-insulin IgG subclass distribution. Spearman's Rank Correlation Coefficient (r_s) values:

	% Suppression					
	H10	H100	P10	P100	B10	B100
N	22	22	32	32	32	32
Anti-Insulin IgG Subclass						
anti-IgG1	-0.012	-0.199	-0.063	-0.132	-0.206	-0.086
anti-IgG2	-0.185	-0.145	-0.076	-0.038	0.087	-0.028
anti-IgG3	0.038	0.229	0.163	0.170	0.179	0.071
anti-IgG4	0.461 *P<0.05*	0.033	0.065	-0.144	0.235	-0.142

N=Number of patients. % Suppression in the presence of H=human; P=pork; and B=beef insulin at 10 and 100ug.ml^{-1}.

Figure 4.6.


RELATIONSHIP BETWEEN ANTI-INSULIN IgG4 ANTIBODY TITER
AND SUPPRESSOR CELL ACTIVITY
% SUPPRESSION
100
90
80
70
60
50
40
30
20
10
0
-10
-20
-30
-40
-50
-60
-70
0
5
10
15
20
25
30
35
ANTI-INSULIN IgG4
% SUPPRESSION WITH 10ug/ml HUMAN INSULIN
N=21
r=0.461
P<0.05

4.2. DISCUSSION

The Relationship Between The Cellular And The Humoral Immune Response To Insulin

The immune system is an intricate balance between cellular components which include antigen presenting cells, T-lymphocytes and components responsible for the humoral response, mediated by B-cells. The purpose of this study was to concentrate on the T- and B-cell response to insulin in IDDM patients. As these two types of responses are integrated it was of interest to investigate the relationship between them.

There was no correlation between T-cell proliferation (measured in terms of stimulation index) and the concentration of total anti-insulin IgG in the sera of diabetic or control subjects. There was, however, an inverse relationship between T-cell proliferation in the presence of $10ug.ml^{-1}$ human and beef insulins and the level of IgM. The level of IgE found in the sera of group I diabetic patients also decreased with increase in SI values obtained when lymphocytes from diabetic patients were cultured in the presence of $100ug.ml^{-1}$ pork insulin. Such associations may reflect the influence of T-cells on the immunoglobulin isotype synthesised by B-cells as well as the amount of IgM and IgE produced by diabetic patients.

The levels of IgG4 and IgE in sera of group I diabetic patients were found to increase with increase in suppressor cell activity (% suppression in the presence of 10ug.ml^{-1} human insulin). Interestingly, all three types of responses (IgG4, IgE and % suppression) increased with increase in insulin dose. These three phenomena appear to be a function of insulin dose. It is possible that in patients who require a high insulin dose, there is a degree of insulin resistance which may be due to IgG4 and IgE antibodies forming complexes with insulin that does not necessarily lead to insulin allergy, but affects the insulin dose. Alternatively, high insulin dose induces increased IgG4 and IgE production. The level of IgG4 and IgE may then trigger a negative feed-back mechanism which activates suppressor cells. Such a hypothesis is purely speculative as there is no evidence to suggest that such complexes are formed.

The levels of IgG4, IgE and together with % lymphocyte suppression (in the presence of 10ug.ml^{-1} human insulin) also decreases with age. The relationship between these factors and age may be part of a general loss of immunity as a result of the ageing process.

There was a direct relatiionship between IgE protein and IgG4 anti-insulin antibodies. i.e. the level of anti-insulin IgG4 antibody increased with an increase in IgE. Increaseed IgG4 has been reported in patients with

allergy and parasitic infections (Oxelius, 1984; Shakib, 1986; Aalberse, et. al., 1983), IgG4 and IgE antibodies appear to be important in allergic diseases and since serum specific IgE antibody responses are influenced by MHC genes (Marsh, et. al., 1981), it is possible that similar genetic restrictions govern the level of anti-insulin IgG4 produced in insulin-treated diabetics. IgG4 is known to be cytophilic for basophils, and heterologous anti-IgG4 antisera can stimulate peripheral blood leucocytes from normal or allergic individuals to degranulate and release histamine (Shakib, 1986). However, anti-insulin IgG4 was found in sera of diabetic patients with no apparent clinical sensitivity to insulin, thus IgG4 cannot be implicated in allergic reactions. The high level of anti-insulin IgG4 found in some patients is therefore, thought to reflect prolonged antigenic stimulation known to elicit IgG4 response (Aalberse et. al., 1983). From an immunopathological point of view, IgG4 antibodies are thought to be harmless, and possibly protective against complement-induced damage (Allberse, et. al., 1983).

In patients with poor diabetic control (high % HbA1), cellular immune response (SI) was found to be higher than in those with good diabetic control (low % HbA1) (Chapter II; Table 2.17). The reverse was true of the humoral response (anti-insulin IgG concentration), which was lower in patients with poor control than in those with good diabetic control (Chapter III; Table 3.8). It

appears that diabetic control may influences the cellular immune response to insulin in some way, although the exact mechanism is unclear. The antibody response on the other hand has a direct affect on diabetic control i.e. in patients with good diabetic control the increased levels of insulin antibody in their sera, acts as a buffering system and prolongs the effects of insulin thus reducing the level of glycosylated haemoglobin.

Finally, a preliminary analysis of the serum interleukin-2 (IL-2) level was determined in 15 Group I diabetic patients and 8 non-diabetic subjects. The results confirm previously reported findings (Kaye, et. al., 1986; Rodman, 1984), i.e. the level of IL-2 found in the sera of diabetic patients were significantly lower than in the sera from normal controls.

Deficient IL-2 production is also reported in other autoimmune diseases such as systamiclupus erythematoses (SLE). The level of IL-2 is reported to be normal in type II diabetic patients (Kaye, et. al., 1986). Rodman, (1984), also established a genetic link, IL-2 deficient Type I diabetics were found to be positive for HLA-DR3, although Kaye, et. al., found no such genetic link. It was not possible to establish any relationship between serum IL-2 concentration and the clinical background of the patients, as the number of patients was too low. Further study with more patients will be necessary in order to investigate the possible relevance of the above

findings to the immune response to insulin.

CHAPTER V

5. DISCUSSION

The cellular and humoral immune responses to insulin in IDDM patients and non-diabetic control subjects were investigated. The work presented in this study largely confirm and extend previously published reports concerning the immunogenicity of exogenously administered human and/or beef insulins.

Cellular immune response to insulin, assessed by the *in vitro* cellular proliferation to human, pork and beef insulins, showed that insulin-specific lymphocyte transformation occurs in approximately 40% of Group I diabetic patients. Nearly all the diabetic patients studied have long established insulin-dependency. It is reasonable to assume therefore, that these patients have developed cellular immunity in the form of an insulin-sensitized lymphocyte subpopulation. Since the level of the stimulation index (SI) was found not to be influenced by the patients' age, duration of disease, duration of insulin therapy, insulin dose, or any immunological complications (such as insulin resistance and insulin allergy), such insulin-sensitized lymphocytes are thought to be of little clinical significance.

Most patients, undergoing human insulin therapy, showed very low stimulation indices (< 2.5), the maximum

response being a SI value of 8.76. Approximately 60% of the Group I patients failed to show a significant SI value. Thus, it is concluded that therapeutic human insulin is not immunogenic in the majority of patients. In patients treated with human insulin from the onset of insulin therapy, it was found to be even less immunogenic.

The level of % glycosylated haemoglobin was found to be elevated in patients who gave a positive response to insulin. The relationship between diabetic control and SI is unclear but it may be associated with the level of anti-insulin antibody. For example, 18 of 24 patients who gave a positive proliferative response to insulin also showed significant levels of insulin antibody in their serum. It is possible that positive responders (ie. those patients who gave a positive SI value) possess insulin sensitized T-cells which influence a B-cell response and the level of insulin antibody produced. Thus patients with insulin sensitized T-cells produce more antibody which positively affect the patient's diabetic control by acting as a buffering agent between insulin injections. Unfortunately, no correlation between SI and anti-insulin IgG concentration was observed.

The proliferative response to insulin was found to be partly regulated at the cellular level by suppressor cell activity. i.e. a negative correlation between SI

and insulin specific % suppression was observed. The immune response to insulin was also reported by others to be controled at the cellular level by suppressor cells (Jensen & Kapp, 1985; Baskin & Rosenthal, 1980). This finding may be of great importance in view of the autoimmune process of type I diabetes.

Insulin-specific suppressor cell activity in the presence of at least one type of insulin was observed in 50 of the 63 patients studied. The remaining 13 patients failed to give positive suppressor cell activity with all three types of insulin in culture. Of these 13 patients, three are known to have diabetes retinopathy, one showed signs of insulin resistance (daily dose >200 units) and one had diabetes neuropathy. Of the 50 patients who did show suppressor cell activity, only four had diabetes retinopathy. It is, therefore, tempting to speculate that lack of insulin specific suppressor cells may have detrimental effects, which results in various complications associated with insulin therapy. Further investigation is obviously required.

The effect of insulin antibody on insulin dose requirement is controversial. Some investigations failed to show a correlation (Asplin, et. al., 1978; Chandraprasert, 1985; Gray, et. al., 1985;), while others (Andersen, 1972; Ludvigasson, 1984; Wajchenberg, et. al., 1986) report that patients with high antibody titers require higher insulin doses. The results

obtained in the present study confirm the former reports.

The presence of insulin antibodies is thought to be detrimental to islet beta cell function (Ludvigsson, 1984), and therefore, may exacerbrate the autoimmune process of the disease. The mechanism is unclear, but it is speculated that insulin antibodies could have an 'exhausting' effect by binding endogenously secreted insulin and proinsulin (Ludvigsson, 1984). Alternatively, insulin antibodies and complexes of antibodies may act as antigens and contribute to inflammatory reactions in the islets of Lagerhans. Because it was not within the scope of this investigation to determine the level of beta-cell function in the diabetic patients studied, it is not possible to confirmed or refuted the above hypothesis.

The importance of insulin antibodies depend on whether they offer clinically significant advantages or disadvantageous to diabetic patients treated with insulin. For example, it is suggested by various investigations that alterations in the pharmacokinetics and localisation of insulin in the presence of insulin antibodies have significant effects on the adequacy of diabetic control (Reeves, 1986; Dixon, et. al., 1975).

In view of the finding that insulin antibody may influence diabetic control, it is important to consider the antibody status of an individual when determining on the most appropriate therapeutic insulin regime. For example, in some patients, with poor diabetic control,

stimulation of the immune system to produce antibody similar to the levels found in patients with good control may be beneficial. It is possible that, in view of the discomfort associated with sudden hypoglycemia experienced by some patients treated with human insulin (which may be associated with low levels of insulin antibodies), some patients may in fact benefit from therapy with the more immunogenic beef insulin rather than with human insulin.

However, it is important to remember that almost all cases of diabetic complications thought to be associated with insulin antibodies have a history of previous treatment with conventional beef insulin preparations.

There was little evidence to suggest that transferring patients from highly purified beef to highly purified human insulin therapy has any suppressive effect on the cellular immune response to insulin. There was, however, a reduction in the serum concentration of total anti-insulin IgG in patients who gave very high antibody titers, and the level of IgE in the serum of Group II patients decreased when they were transferred to human insulin therapy. Since insulin allergy is associated with beef insulin therapy rather than human insulin (Reeves, 1980; 1986), the decrease in IgE, when transferred to human insulin therapy, may be due to the likelihood that human insulin is less immunogenic than

its beef counterpart.

The most interesting findings made in this study were related to anti-insulin IgG subclass distribution. Insulin therapy seems to preferentially stimulate IgG1 and IgG3 subclasses with the levels of IgG2 and IgG4 varying considerably from patient to patient. This contrasts with the normal distribution of total IgG subclasses, which was 59.9% IgG1, 18.3% IgG2, 9.2% IgG3 and 12.5% IgG4.

Increased levels of anti-insulin IgG4 was found in some cases and was correlated to insulin dose, thus it may be associated with chronic antigenic stimulation. The level of anti-insulin IgG4 antibodies and IgE protein was found to increase with % suppression (at $10ug,ml^{-1}$ human insulin). All three factors appear to be a function of insulin dose. There was strong correlations between % suppression, IgG4 and IgE, all of which decreased with age. The decrease in IgG4, IgE, and insulin-specific % suppression, may all be part of the side-effects of the ageing process.

The level of anti-insulin IgG2 was found to be very low or absent in some diabetic patients. IgG2 deficiency is thought to be associated with autoimmune diseases including systemic lupus erythematosus (SLE) and insulin-dependent diabetes mellitus (Shakib, 1986; Oxelius, 1984; Koch, et. al., 1986). Deficiency in IgG2 and IgG4 are also reported to be associated with IgA

deficiency.

Thus, insulin appears to stimulate its own characteristic pattern of IgG subclasses with IgG1 and IgG3 being predominant in most patients. The level of IgG4 and IgG2 varied considerably from patient to patient and appears to be influenced by the patients' daily insulin dose and their age. The significance of the increased level of IgG4 in some patients, and the absence of IgG2 in others is not clear but such restrictions are thought to be due to the antigenic nature of insulin and the prolonged duration, and route of immunization.

In conclusion, human insulin therapy is immunologically sound as judged by the low level of SI values which were obtained in only 40% of patients. The antibody response was equally low as only 4 patients, of the 76 studied, gave high anti-insulin IgG titers. Human insulin is probably less immunogenic than beef insulin, especially in patients with high antibody titers. Patients treated with human insulin from the onset of insulin therapy showed little cellular and humoral responses. It would be interesting to expand this study to include a greater number of patients on human insulin and determine the immunological effects of human insulin therapy only. Since genetic factors may be involved it may be of interest to determine the HLA background of these patients and perhaps establish any genetic link present.

7. BIBLIOGRAPHY

Aalberse. R.C., van der Gaag. R., van Leeuwen. J. (1983). 'Serologic aspects of IgG4 anitbodies - Prolonged Immunisation Results in an IgG4-Restricted Response'. J. Immunology 130 No.2: 722-726.

Almer. L.O., Ekberg. G., Fankhauser. S., Home. P.D., Worth. R., Sailer. S., Kurtz. A.B., and Christy. M., (1985). 'A prospective study of the immunogenicity of porcine insulin in HLA-typed new insulin-treated diabetics.' Diabetes Research 2:221-224.

Anderson. O.O., (1972), 'Insulin Antibody Formation - the influence of age, sex. infections. insulin dosage and regulation of diabetes.' Acta Endocrinol.. 71:126-140.

Anderson. O.O., (1976). 'Anti-insulin-antibodies and late diabetic complications.' Acta. Endocrinol. 83:329-340.

Asplin. C.M., Hartog. M., Goldie. D.J. (1978). 'The relationship between circulating free and bound insulin. insulin antibodies. insulin dosage and diabetic control in insulin treated diabetics.' Acta Endocrinol. 87:330-338.

Asplin, C.M., Dornan. T.L., Raghu. P.K., Hansen. J.A., Palmer. J.P, (1984). 'The antibody response to insulin therapy. A prospective study in HLA-typed. insulin-dependent diabetic subjects.' Diabetc 33:966-969.

Baratono. S., Tovo. P., Cerutti. F., Beatrice. M., Palomba. E., Pugliese. A. (1980). 'An acid-labile interferon in insulinopenic diabetes.' Diabetologia 19:255 (Abstr.).

Baskin, B.L., and Rosenthal. A.S., (1980). 'Determinant specific suppression of antigen-induced T-cell proliferation in the guinea pig - I. Quantitation of suppressed antigen-specific T-cell responses as a consequence of prior exposure to antigen in incomplete Freud's Adjuvant'. J. Immunol. 124 No.1:184-193.

Berson. S.A., Yalow. R.S., Bauman. A., Rothschild. M.A.,

Newerly. K. (1956). 'Insulin I131 metabolism in human subjects. Demonstration of insulin binding globulin in circulation of insulin treated subjects.' J. Clin. Invest. 35:170 .

Bloom, S.R., Adrian, T.E., Barnes. A.J., Polak. J.M., (1979). 'Auto-immunity in diabetics induced by hormonal contaminants of insulin. Lancet 1:14.

Blundell. T.L., Cutfield. J.F., Cutfield. S.M., Dodson. E.J., Dodson. G.G., Hodgkin. D.C., Mercola. D.A. (1972). 'Three-Dimensional Atomic Structure of Insulin and its relationship to activity.' Diabetes 21 (suppl 2):492-505.

Boyum. (1968). 'Isolation of mononuclear cells and granulocytes from human blood. Isolation of mononuclear cells by one centrifugation, and of granulocytes by combinating centrifugation and sedimentation at 1 g. Scand. J. Clin. Lab. Invest. 21 Suppl. 97:51-76.

Buchard. K., Madsbad. S., Krarup. T., Rygaard. J, (1982). 'Glycaemic control and suppressor cell activity in patients with insulin-depenednt diabetes mellitus.' Clin. exp. Immunol. 48:189-195.

Buschard. K., Madsbad. S., Rygaard. J., (1980). 'Depressed suppressor cell activity in patients with newly diagnosed insulin-dependent diabetes mellitus. Clin. exp. Immunol. 41:25 .

Catt. K., and Tregear. G.W., (1976). Science 158:1570-1571 .

Chandraprasert. S., and Bunnag. S.C., (1985). 'Insulin Antibodies In Diabetics After Changeover From Conventional NPH To Highly Purified Insulin' Ann. acad. med. Singapore. 14 No.2:289-293.

Damle. N.K., Mohagheghpour. N., Hansen. J.A., and Engman. G. (1983). 'Alloantigen-specific cytotoxic and suppressor T-lymphocytes are derived from phenotypically distinct precursors.' J. Immunol. 131 No.5:2296-2300 .

Di Mario. U., Arduini. P., Tiberti. C., Lombardi. G., Pietravalle. P., Andreani. D. (1986). 'Immunogenicity of biosynthetic human insulin - Humoral immune response in diabetic patients begining insulin treatment and in patients previously treated with other insulins.' Diabetes Res. & Clin. Practice. 2:317-324.

Diem. P., Spengler. H., De Week. A.L, (1982). 'Lymphocyte transformation by insulin and insulin-zinc suspensions.' Clin. exp. Immunol. 50:155-162.

Dixon. K., Exon. P.D., Malins. J.M., (1975). 'Insulin Antibodies and the Control of Diabetes'. Quaterly J. Med., New Series. XLIV. No. 176:543-553.

Erb. P, (1980). 'Analysis of the in vitro immune response to insulin - 1. Primary induction of insulin-specific T helper cells and characterisation of the genetic control of the helper cell response to bovine and porcine insulins.' Immunology, 40:385-393.

Glimcher. L.H., Schroer. J.A., Chan. C., Sheyach. E.M, (1983). 'Fine specificity of cloned insulin-specific T cell hybridomas: Evidence supporting a role for tertiary conformation.' J. Immunol. 131 No.6:2868-2874.

Goldman. B.C.J., Baldwin.D., Goldberf. R.B., Ryan. W.G., Blix. P.M., Schanslin, D., Fritz. K.J., Rubenstein. A.H. (1979) 'Metabolic Control in Diabetic Patients - Effect of Insulin-secretory Reserve (Measured by Plasma C-peptide Levels) and Circulating Insulin Antibodies'. Diabetes 28:749-753.

Gray. R.S., Borsey. D.Q., Kurts. A., Rainbow. S., Smith. A.F., Elton. R.A., Duncan. L.J.P., Clarke. B.F, (1981). 'Relationship of Glycosylated Haemoglobin to C-peptide secretory status and antibody binding of insulin in insulin-dependent diabetes.' Horm.Metab. Res. 13:599-603.

Gray. R. S., Cowan. P., Di Mario. U., Elton. R.A., Clarke. B.F., Duncan. L.J, (1985). 'Influence of insulin antibodies on pharmacokinetics and bioavailability of recombinant human and highly purified beef insulins in insulin dependent diabetics.' Brit. Med. J. 290:1687-1691.

Herold. K.C., Rubenstein. A.H., (1988). 'Immunosuppression for insulin-dependent diabetes'. New Eng. J. Med. 318 No.11:701-703.

Hitman, G.A., Tarn, A.C., Winter. R.M., Drummond. V.,

Williams. L.G., Jowett. N.I., Bottazzo. G.F., Galton. D.J. (1985). 'Type I (insulin-dependent) diabetes and a highly variable locus close to the insulin gene on chromosome II.' Diabetologia 28:218-222.

Horowits. S.D.. Borcherding. W., Bergman. G.J. (1977). 'Suppressor T-cell function in diabetes mellitus'. The Lancet 17:1291 .

Ionescu-Tirgoviste. C., Mincu. I., Simionescu. L., (1984). 'Disappearance rate of insulin antibodies after discontinuing insulin treatment in 42 Type 2 (non-insulin-dependent) diabetic patients.' Diabetologia. 27(6):592-595.

Irvine. J. (ed) (1980). 'Immunology of diabetes' Pub. Teviot Scientific Publications. Edinburgh. U.K. ISBN 09 06341 019.

Jefferis. R., Reimer. C.B., Skvaril. F., de Lange. G., Ling. N.R., Lowe. J., Walker. M.R., Phillips. D.J., Aloisio. C.H., Wells. T.W., Vaemerman. J.P., Magnusson. C.G., Kubagawa. H.. Cooper. M., Vartdal. F., Vandvik. B., Haaijman. J.J., Makela. O., Sarnesto. A., Lando. Z., Gergely. J., Rajnavolgyi. E.. Laszlo. G., Radl. J., Molinaro. G.A. (1985). 'Evaluation of monoclonal antibodies having specificity for human IgG sub-classes: results of an IUIS/WHO collaborative study' Immunol. Letters 10:223-252.

Jensen. P.E., Kapp. J.A. (1985). 'Genetics of insulin-specific helper and suppressor T cells in nonresponder mice.' J. Immunol. 135 No.5:2990-2995.

Karjalainen. J., Salmela. P.. Ilonen. J., Surcel. H.M., Knip. M. (1989). 'A Comparison of Childhood and Adult Type I Diabetes Mellitus.' New Eng. J. Med. 320 No.14:881-886.

Kaye. W.A., Adri. M.N.S., Soeldner. J.S., Rabinowe. S.L., Kaldany. A., Kahn. C.R., Bistrian. B., Srikanta. S., Ganda. O.P., Eisenbarth. G.S. (1986) 'Acquired defect in interleukin-2 production in patients with type I diabetes mellitus.' New Engl. J. Med. 315 No. 15:920-924.

Keilacker. H., Rjasanowski. I., Ziegler. M., Michaelis. D., Woltanski. K.P., Besch. W. (1982) 'Insulin Antibodies in Juvenile Diabetes Mellitus - Correlations to Diabetic Stability, Insulin Requirement and Duration of Insulin Treatment'. Horm. metabol. Res. 14:237-232.

Keilacker. H., Rjasanowski. I., Ziegler. M., Michaelis. D., Woltanski. K.P., Besch. W. (1986). 'The role of Insulin Antibodies in Insulin Treatment of Type I Diabetes.' Exp. Clin. Endocrinol. 87 No.1:79-88.

Keilacker. H., Rjasanowski. I., Ziegler. M., Michaelis. D., Oxelius. V., (1984).'Immunologlobulin G (IgG) Subclasses and Human Disease'. American J. Medicine. March 30 : 7-18.

Koch. M., Francois-Gerald. C., Sodoyes-Goffaux. F., Sodoyes. J.C. (1986). 'Semi-quantitative assessment of anti-insulin total IgG and IgG sub-classes in insulin-immunized patients using a highly sensitive immunochemical micromethod.' Diabetologia. 29:720-726.

Kontiainen. S. (1982). 'Human insulin specific suppressor factors - I. Induction. characteristics and comparison to murine factors.' Clin. exp. Immunol. 47:715-724.

Kynoch, P.A., Lehmann. H. (1977). 'Rapid estimation (2.5 hours) of glycosylated haemoglobin for routine purposes'. Lancet 2(8027):16.

Ludvigasson. J., (1984). 'Insulin antibodies in diabetic children treated with monocomponent procine insulin from the onset: relationship to B-cell function and partial remission' Diabetologia 26:138-141.

Lunetta. M., Leonardi. R., . Sudano. L. (1986). 'Antigenicity of semisynthetic human insulin in newly diagnosed type 1 diabetic patients: difference between short and intermediate acting preparations.' Diatetes & Metabolism (Paris) 12:83-84.

MacCuish. C.J., Jordan. J., Campbell. C.J., Duncan. L.J.P., Irvine. W.J. (1975). 'Cell-mediated Immunity in Diabetes Mellitus - Lymphocyte Transformation by Insulin and Insulin Fragments in Insulin-treated and Newly-diagnosed Diabetics.' Diabetes. 24 No.1:36-43.

McEvoy. R.C., Witt. M.E., Cinsberg-Pellner. P., Rubinstein. P. (1986). 'Anti-Insulin Antibodies in Children With Type I Diabetes Mellitus - Genetic Regulation of Production and Presence at Diagnosis Before Insulin Replacement.' Diabetes. 35:634-641.

Mann. D.L., Mendell. N., Kahn. C.R., Johnson. A.H., Rosenthal, A. (1983). 'In Vitro Lymphocyte Proliferation Response to Therapeutic Insulin Components.' J. Clin. Invest. 72:1130-1138.

Marsh. D.G., Meyers. D.A., Bias. W.B., (1981). 'The epidemiology and genetics of atopic allergy.' New Engl. J. Med. 305:1551-1559.

Marx, J.L., (1975). 'Suppressor T-cells: Role in immune regulation.' Science 188:245-247.

Miller. G.G., Pollack. M.S., Nell. L.J., Thomas. J.W, (1987). 'Insulin-specific Human T Cells - Epitope Specificity. Major Histocompatitibility Complex Restriction and Alloreactivity to a Diabetes-Associated Haplotype.' J. Immunol. 139 No.11:3622-3629.

Moore. K., Nesbitt. A.M., (1986). 'Identification and isolation of OKT4+ suppressor cells with the monoclonal antibody WR16'. Immunol. 58:659-664.

Moore. K., Nesbitt. A.M., (1987). 'Functional heterogeneity of CD4+ T-lymphocytes: two subpopulations with counteracting immunoregulatory functions identified with monoclonal antibodies WR16 and WR19'. Immunol. 61:159-165.

Nakao, Y., Matsumoto. H., Miyazaki. T., Mizuno. N., Arima. N., Wakisaka. A., Okimoto. K., Akazawa. Y.. Tsuji. K., Fujita, T, (1981). 'IgG Heavy-Chain (Gm) allotypes and immune response to insulin in insulin-requiring diabetes melittus.' New Eng. J. Med. 304(7):407-409.

Naquet. P.. Ellis. J., Tibenski. D.. Kenshole. A., Singh. B.. Hodges. R., Delovitch. T.L. (1988). 'T Cell Autoreactivity To Insulin In Diabetic And Related Non-Diabetic Individuals.' J. Immunol. 140 No.8:2569-2578.

Nell, L.J., Thomas. J.W. (1983). 'The Human Immune response To Insulin - Kinetic and Cellular Aspects of Lymphocyte Proliferative Response in Diabetics.' J. Immunol. 131 No.2:701-705.

Nell. L.J., Virta. J., Thomas. J.W. (1985). 'Recognition of Human Insulin In Vitro by T Cells from Subjects Treated with Animal Insulins.' J. Clin. Invest. 76:2070-2077.

Notkins. A.L., (1979). 'The cause of diabetes.' Sci. Ar 241:62 .

Page-Faulk. W., Karam. J.H., Fudenberg. H.H, (1971). 'Human Anti-insulin Antibodies.' J. Immunol. 106 No. 4:1112-1116.

Owerbatch. D., Lernmark A., Platz. P., Ryder. L.P., Rask. L., et. al., (1983). 'HLA-DR Beta-chain DNA endonuclease fragments differ between healthy and insulin-depedent diabetic individuals.' Nature 303u:315 .

Palmer. J.P, (1987). 'Insulin Autoantibodies:Their role in the Pathogenesis of IDDM'. Diabetes Metabol. Rev. 3No. 4:1005-1015.

Parker. B.A.. Reeves. W.G. (1989). 'In vitro priming of human lymphocytes to heterologous insulins.' J. Immunol. Methods. 120:159-165.

Pickup. J., (1986) 'Human Insulin'. Brit. Med. J. 292 No. 6514:155-157.

Reeves. W.G., (1980). 'Immunology of diabetes and insulin therapy'. Recent advances in clinical Immunology. Ed. by Thomson. pub. Churchill-Livingstone. Edinbrough. p.183-219.

Reeves. W.G., Barr. D., Douglas., C.A., Gelsthorpe. K., Hanning. I., Dkene. A., Wells. L., Wilson. R.M., Tattersall. R.B, (1984). 'Factors governing the human immune response to injected insulin'. Diabetologia. 26:266-271.

Reeves. W.G., (1985). 'Immunogenicity of insulin of various origins'. Meth. J. Med. 28. suppl. 1:43-16.

Reeves. W.G., (1986). 'The immune response to insulin: characterisation and clinical consequences'. Diabete Annual. 2:81-93.

Rodboard. D., Hutt, D.M., (1974). 'Statistical analysis of radioimmunoassays and immunoradiometric (labelled antibody) assays: a generalized, weighted, least square method for logistic curve fittings'. In symposium on RIA and related procedures in medicine. IAEA. Vienna. page 165-192.

Rodman. H.M., (1984). 'Deficient Interleukin-2 synthesis in type 1 diabetes'. Abstract Diabetes 33:suppl.1:11A .

Rogala. H.. Lawecki. J., Czyzyk.A. (1986) 'Immunogenicity of semisynthetic human insulin'. Acta Med. Pol. 27:1-2.

Rosenwasser, L.J., Barcindki, M.A., Schwarts. R.H., Rosenthal. A.S. (1979). 'Immune Response Gene control of determinant selection - Genetic control of the murine T-lymphocyte proliferative response to insulin'. J. Immunol. 123 No.1:471-475.

Rossini, A.A., Mordes. J.P. and Like. A.A. (1985). 'Immunology of Insulin-dependent Diabetes Mellitus'. Ann. Rev. Immunol. 3:289-320 .

Scheinin. T.. Groop, L., Mikkila. E.. Kontiainen. S. (1983). 'Proliferative Responses To Insulin Antigens In Diabetics And Controls.' J. Clin. Lab. Immunol. 11:135-141.

Schernthaner, G., Ludeig. H:, Mayr. W.R, (1979). 'Immunoglobulin G-Insulin Antibodies and Immune Region-Associated Alloantigens in Insulin-Dependent Diabetes Mellitus.' J. Clin. Endocrinol. & Metabol. 48(3):403-407 .

Schernthaner. G. (1982). 'Affinity of IgG-insulin Antibodies to Human (recombinant DNA) Insulin and Porcine insulin in Insulin-treated Diabetic Individuals with and without insulin resistance'. Diabetes Care 5 Suppl. 2:114-128 .

Schroer. J.A., Bender, T., Feldmann. R.J., Kim. K.J., (1983). 'Mapping epitopes on the insulin molecule using monoclonal antibodies.' Eur. J. Immunol. 13:693

Scott. D.A., Fisher. A.M., (1986). 'Studies on insulin with protamine.' J. Phaarmacol. & Expt. Therapeutics. 58:78.

Sklenar, I., Neri, T.M., Berger, W., Erb, P. (1982). 'Association of specific immune response to pork and beef insulin with certain HLA-DR antigens in type I diabetes'. Brit. Med. J., 285:1451-1453.

Shakib, F. (1986). Editor. 'Basic and Clinical Aspects of IgG Subclasses'. Monographs in Allergy, vol. 19. Pub. by Karger. Basel.

Stinmetz, M., Hood, C., (1983). 'Genes of the major histocompatibility complex in mouse and man.' Science 222:727 .

Unanue, E.R., Beller, D.I., Lu, C.Y., Allen, P.M. (1984). 'Antigen Presentation: Comments on its Regulation and Mechanism.' J. Immunol. 132 No.1:1-5.

Wajchemberg, E.L., Thomsen, Y.G., Toledoe Souza, I.T., Germek, O.A. (1986). 'Comparison of insulin antibody levels during the first 3 years of treatment of adult deabetics with monocomponent procine lente-insulin and single peak beef NPH insulin'. Horm. Metabol. Res. 18:535-539.

Walford, S., Allison, S.P., Reeves, W.G. (1982). 'The Effect of Insulin antibodies on Insulin Dose and Diabetic Control.' Diabetologia. 22:106-110.

Walford, S., (1985) 'Insulin antibodies - do they matter' Meth. J. Med. 28. suppl. 1:47.

Wardlaw, A.C., (1987). "Practical Statistics for Experimental Biologist". John Wiley & Sons. Wiley-InterScience Publication.

Williams, R.C., Messner, R.P. (1975). 'Alterations in T and B cells in human disease states.' Annual. Rev. Med. 26:181-192.

Wilson, R.M., Douglas, C.A., Tattersall, R.B., Reeves, W.G., (1985). 'Immunogenicity of highly purified bovine insulin: a comparison with conventional bovine and highly purified human insulins.' Diabetologia 28:667-670.

Wolf, E., Spencer, K.M., Cudworth, A.C., (1983). 'The genetic susceptibility to type I (insulin-dependent)

diabetes: analysis of the HLA-DR association.' Diabetologia 24:224.

Wysocki, L.J., Sato, V.L., (1978). ''Panning' for lymphocytes: A method for cell selection'. Proc. Natl. Acad. Sci. USA 75 No. 6: 2844-2848.

Yagi, Y., Maier, P., Pressman, D. (1962). 'Two different anti-insulin antibodies in guinea pig antisera.' J. Immunol. 89:442-451.